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The signaling lipid sphingosine 1-phosphate regulates mechanical pain

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Abstract

Somatosensory neurons mediate responses to diverse mechanical stimuli, from innocuous touch to noxious pain. While recent studies have identified distinct populations of A mechanonociceptors (AMs) that are required for mechanical pain, the molecular underpinnings of mechanonociception remain unknown. Here, we show that the bioactive lipid sphingosine 1-phosphate (S1P) and S1P Receptor 3 (S1PR3) are critical regulators of acute mechanonociception. Genetic or pharmacological ablation of S1PR3, or blockade of S1P production, significantly impaired the behavioral response to noxious mechanical stimuli, with no effect on responses to innocuous touch or thermal stimuli. These effects are mediated by fast-conducting A mechanonociceptors, which displayed a significant decrease in mechanosensitivity in S1PR3 mutant mice. We show that S1PR3 signaling tunes mechanonociceptor excitability via modulation of KCNQ2/3 channels. Our findings define a new role for S1PR3 in regulating neuronal excitability and establish the importance of S1P/S1PR3 signaling in the setting of mechanical pain thresholds.

Research organism: Mouse

Introduction

Pain is a complex sensation. It serves to protect organisms from harmful stimuli, but can also become chronic and debilitating following tissue injury and disease. Distinct cells and molecules detect noxious thermal and mechanical stimuli. Thermal pain is detected by thermosensitive TRP channels in subsets of nociceptors ([Caterina et al., 2000](#); [Vriens et al., 2011](#)), and gentle touch is detected by Piezo2 channels in low-threshold mechanoreceptors (LTMRs) ([Ranade et al., 2014](#); [Woo et al., 2014](#)). A δ high-threshold mechanoreceptors (HTMRs) have been shown to play a key role in responses to painful mechanical stimuli ([Arcourt et al., 2017](#); [Ghitani et al., 2017](#)).

Recent studies have shown that there are at least two populations of HTMRs that mediate responses to noxious mechanical stimuli. The *Npy2r⁺* subpopulation of HTMRs mediates fast paw withdrawal responses to pinprick stimulation and terminates as free nerve endings in the epidermis ([Arcourt et al., 2017](#)). The *Calca⁺* subpopulation of circumferential-HTMRs responds to noxious force and hair pulling, and terminates as circumferential endings wrapped around guard hair follicles ([Ghitani et al., 2017](#)). Additionally, somatostatin-expressing interneurons of laminae I-III in the dorsal horn of the spinal cord receive input from nociceptors and are required for behavioral responses to painful mechanical stimuli ([Duan et al., 2014](#)). Despite these advances in defining the cells and circuits of mechanical pain, little is known about the molecular signaling pathways in mechanonociceptors.

Here, we show that sphingosine 1-phosphate (S1P) is required for mechanical pain sensation. S1P is a bioactive lipid that signals via 5 G-protein coupled S1P Receptors (S1PRs 1–5). S1P signaling, mainly via S1PR1, plays a well-known role in immune cell migration and maturation ([Spiegel and Milstien, 2003](#); [Matloubian et al., 2004](#); [Schwab et al., 2005](#)). Additionally, recent studies have shown that S1PRs are expressed throughout the nervous system ([Janes et al., 2014](#); [Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#)) and S1P signaling is associated with a variety of neuroinflammatory disorders, including multiple sclerosis ([Brinkmann et al., 2010](#)) and Alzheimer's disease ([Couttas et al., 2014](#)). S1P has been implicated in spontaneous pain ([Camprubí-Robles et al., 2013](#)) and thermal pain hypersensitivity ([Mair et al., 2011](#); [Finley et al., 2013](#); [Weth et al., 2015](#)), but due to conflicting accounts of S1P receptor expression in the CNS ([Janes et al., 2014](#); [Weth-Malsch et al., 2016](#)) and PNS ([Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#); [Usoskin et al., 2015](#)) as well as inconsistent reports on the effects of S1P on neuronal excitability ([Camprubí-Robles et al., 2013](#); [Zhang et al., 2006](#); [Li et al., 2015](#)) and pain behaviors ([Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#); [Finley et al., 2013](#); [Weth et al., 2015](#)), the role of S1P in somatosensation remains controversial.

We found that mice lacking the S1P receptor S1PR3 display striking and selective deficits in behavioral responses to noxious mechanical stimuli. Likewise, peripheral blockade of S1PR3 signaling or S1P production impairs mechanical sensitivity. We show that S1P constitutively enhances the excitability of A mechanonociceptors (AMs) via closure of KCNQ2/3 potassium channels to tune mechanical pain sensitivity. The effects of S1P are completely dependent on S1PR3. While previous studies have shown that elevated S1P triggers acute pain and injury-evoked thermal sensitization ([Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#)), we now demonstrate that baseline levels of S1P are

necessary and sufficient for setting normal mechanical pain thresholds. By contrast, elevated S1P selectively triggers thermal sensitization via activation of TRPV1⁺ heat nociceptors, with no effect on mechanical hypersensitivity. Our findings uncover an essential role for constitutive S1P signaling in mechanical pain.

Results

To identify candidate genes underlying mechanosensation, we previously performed transcriptome analysis of the sensory ganglia innervating the ultra-sensitive tactile organ (the star) of the star-nosed mole ([Gerhold et al., 2013](#)). Immunostaining revealed the tactile organ is preferentially innervated by myelinated A δ fibers ([Gerhold et al., 2013](#)), which are primarily mechanosensitive. While our original analysis focused on ion channels enriched in the neurons of the star organ, our dataset also revealed enrichment of several components of the S1P pathway, including *Slpr3*. Likewise, single-cell RNA seq of mouse dorsal root ganglion (DRG) neurons revealed *Slpr3* expression in a subset of myelinated mechanoreceptors ([Usoskin et al., 2015](#)) in addition to a subpopulation of peptidergic C nociceptors.

S1P promotes excitability in small-diameter, capsaicin-sensitive nociceptors ([Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#); [Zhang et al., 2006](#); [Li et al., 2015](#)). In addition, S1PR3 has been shown to mediate spontaneous pain triggered by elevated S1P and thermal sensitization following sterile tissue injury ([Camprubí-Robles et al., 2013](#)). However, no studies have examined the role of S1PR3 in mechanosensation or in regulating somatosensory behaviors under normal conditions. Given the enrichment of *Slpr3* in mechanosensory neurons of the star-nosed mole and mouse, we hypothesized that S1P signaling via S1PR3 may also play a role in mechanosensation. Thus, we set out to define the role of S1P signaling and S1PR3 in somatosensory mechanoreceptors.

S1PR3 mediates acute mechanical pain

We first examined a variety of somatosensory behaviors in mice lacking S1PR3 ([Kono et al., 2004](#)) (*Slpr3*^{tm1Rlp/Mmnc}; referred to herein as S1PR3 KO). We initially investigated baseline responses to mechanical stimuli. S1PR3 KO mice displayed a dramatic loss of mechanical sensitivity ([Figure 1A](#); see [Figure 1—source data 1](#)), as von Frey paw withdrawal thresholds were significantly elevated in S1PR3 KO mice relative to WT and S1PR3 HET littermates (mean thresholds: 1.737 g vs. 0.736 and 0.610 g, respectively). Moreover, S1PR3 KO mice demonstrated decreased responses to a range of noxious tactile stimuli (2–6 g; [Figure 1B](#)) and to noxious pinprick stimulation ([Figure 1C](#)), but normal responsiveness to innocuous tactile stimuli (0.6–1.4 g; [Figure 1B](#)). S1PR3 KO mice exhibited normal tape removal attempts ([Ranade et al., 2014](#)) ([Figure 1D](#)), righting reflexes ([Figure 1—figure supplement 1A](#)), radiant heat withdrawal latencies ([Figure 1E](#)), and itch-evoked scratching ([Figure 1—figure supplement 1B](#)). These results demonstrate a selective role for S1PR3 in acute mechanical pain.

Figure 1. S1PR3 mediates acute mechanical pain.

(A) von Frey 50% withdrawal threshold measurements for *Slpr3*^{+/+} (WT, N = 8), *Slpr3*^{+/-} (HET, N = 7) and *Slpr3*^{-/-} (KO, N = 12) mice. p<0.0001 (one-way ANOVA). Tukey-Kramer post hoc comparisons for KO and HET to WT indicated on graph. **(B)** von Frey force-response graph for WT (N = 8) versus KO (N = 12) animals; $p_{genotype} < 0.0001$ (two-way ANOVA). Tukey HSD comparisons between genotypes are indicated for given forces. **(C)** % withdrawal to pinprick stimulation of hindpaw for HET versus KO animals; p<0.0001 (unpaired t-test; N = 5–7 mice per group). **(D)** Number of attempted removal bouts in tape assay for WT (N = 2), HET (N = 2), and KO (N = 5) mice; p=0.172 (one-way ANOVA). **(E)** Baseline radiant heat measurements for WT (N = 8), HET (N = 3), and KO (N = 5) mice; p=0.444 (one-way ANOVA). **(F)** von Frey 50% withdrawal threshold measurements for mice pre- and post-injection of 500 μM TY 52156 (N = 10), 10 μM W146 (N = 6), or 1% DMSO-PBS vehicle (N = 17); p=0.016, 0.650 (two-tailed paired t-test comparing vehicle- vs. drug-injected paw). **(G)** von Frey force-response graph for mice injected with either 1% DMSO-PBS (N = 4) or 500 μM TY 52156 (N = 4); $p_{treatment} < 0.0001$ (two-way ANOVA). Tukey HSD comparisons were made between treatment groups and significant differences at a given force are indicated on graph. Error bars represent mean ± SD.

Figure 1—source data 1. S1PR3 mediates acute mechanical pain.

Related to [Figure 1](#).

[eLife-33285-fig1-data1.xlsx](#) (42.7KB, xlsx)

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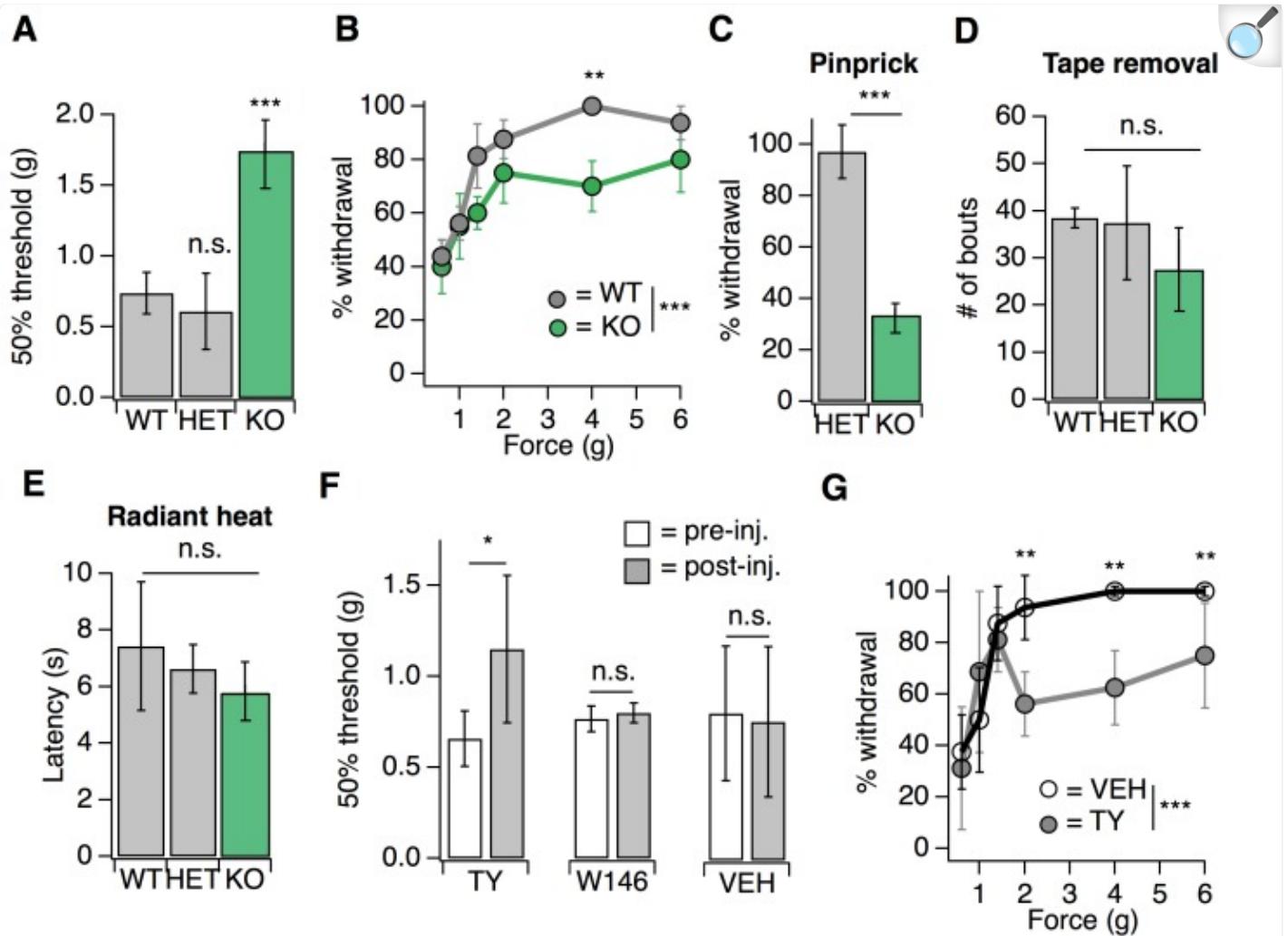
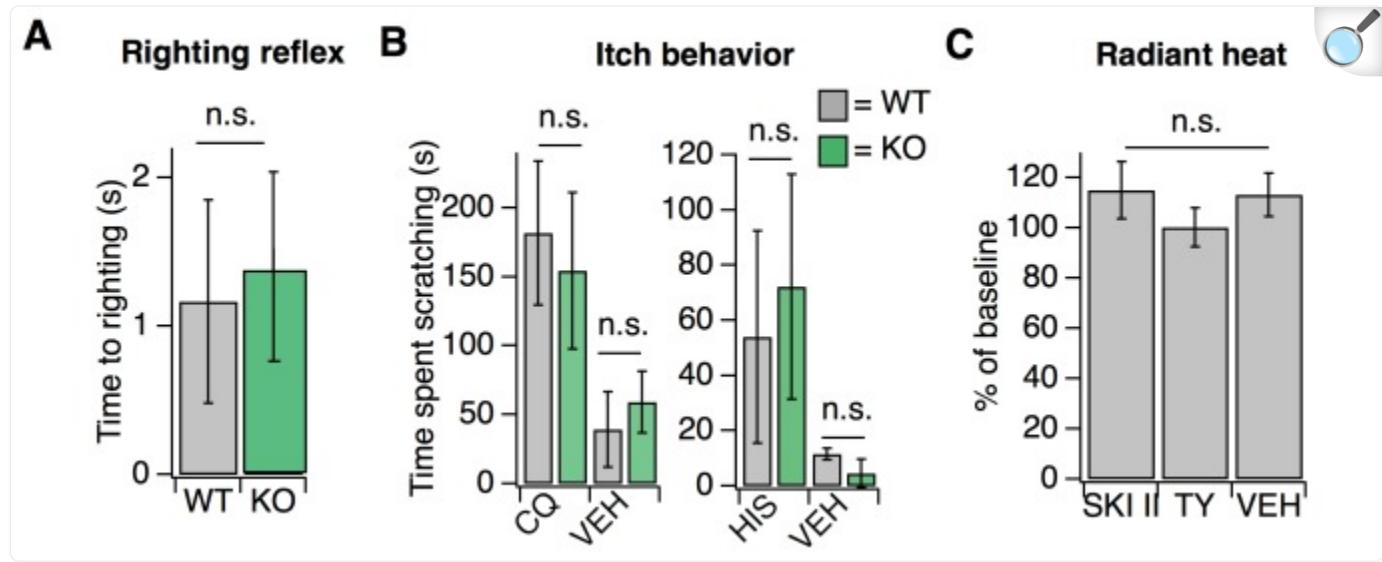


Figure 1—figure supplement 1. Loss of S1PR3 selectively impairs mechanonociception.



Related to [Figure 1](#). (A) Time to righting in seconds for P7 pups per genotype for WT and KO mice; $p=0.575$ (two-tailed unpaired t-test; $N = 7$ mice per genotype). (B) (Left) Time spent scratching in response to injection of 50 mM chloroquine or PBS vehicle (VEH) in WT and KO mice; $p=0.36, 0.98$, (unpaired t-tests; $N = 3–4$ mice per group). (Right) Time spent scratching in response to injection of 27 mM Histamine or 0.1% DMSO-PBS in WT and KO mice; $p=0.51, 0.06$ (unpaired t-tests; $N = 3–4$ mice per group). (C) Normalized paw withdrawal latencies post-injection of SKI II, TY 52156, or 0.1% DMSO-PBS vehicle into the hind paw of wild-type animals; $p=0.65$ (one-way ANOVA; $N = 5$ mice per group). Unless otherwise indicated, error bars represent mean \pm SD.

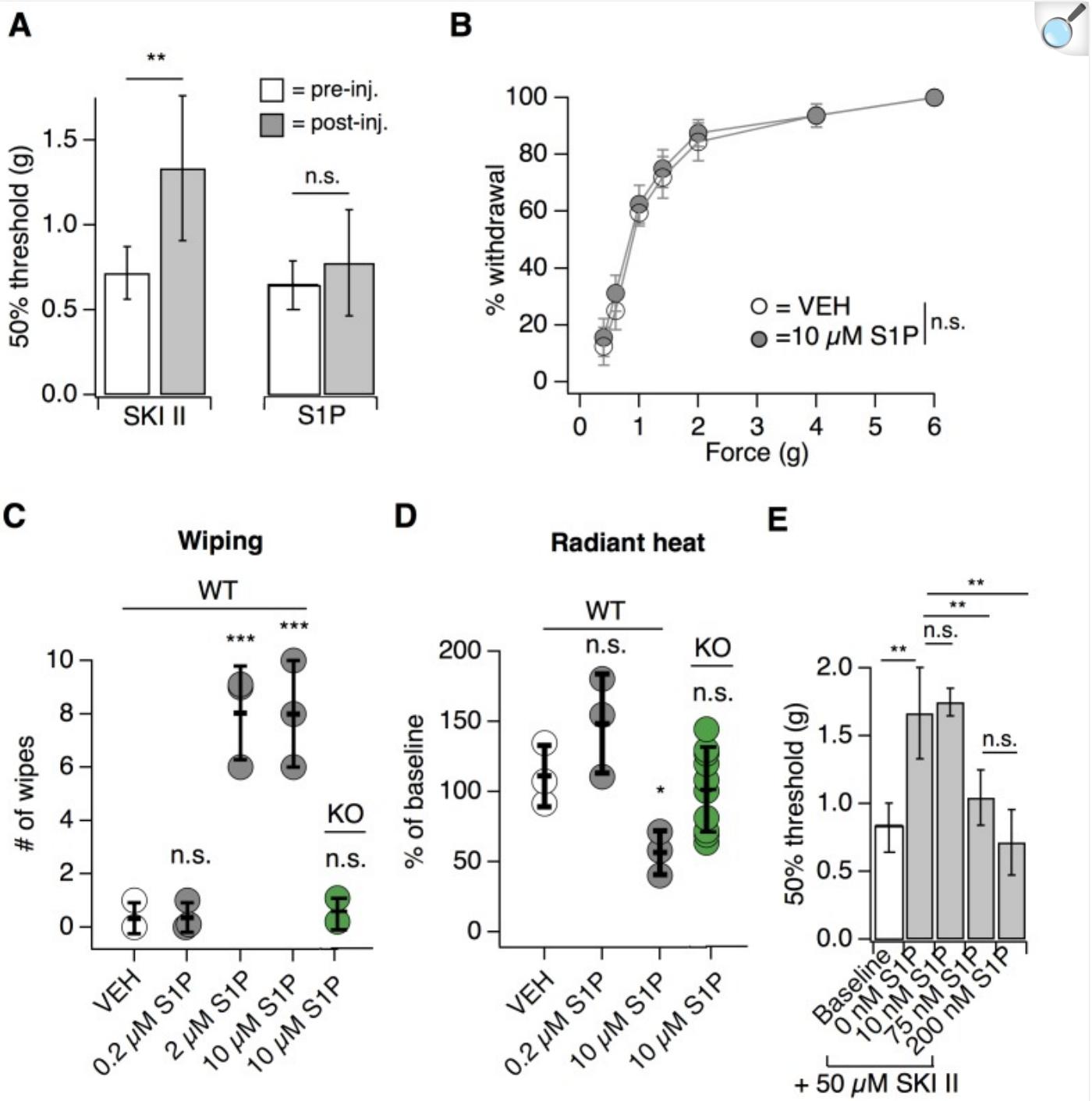
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As a complement to our analysis of somatosensation in S1PR3 KO animals, we employed a pharmacological approach, using the S1PR3-selective antagonist TY 52156 (TY) ([Nussbaum et al., 2015](#)). Similar to the phenotype of knockout animals, intradermal injection of 500 μ M TY into the mouse hindpaw (the site of testing) triggered a rapid and significant elevation in von Frey paw withdrawal thresholds ([Figure 1F](#)) and decreased responsiveness to noxious (2–6 g), but not innocuous (0.6–1.4 g), tactile stimuli ([Figure 1G](#)), without affecting noxious heat sensitivity ([Figure 1—figure supplement 1C](#)). By contrast, blockade of S1PR1 with the selective antagonist W146 ([Finley et al., 2013](#)) had no effect on baseline mechanical or thermal thresholds ([Figure 1F](#); [Figure 1—figure supplement 1C](#)). Overall, these data show that S1PR3 signaling sets mechanical pain sensitivity.

Endogenous S1P mediates acute mechanical pain

We next asked whether peripheral S1P was required for the S1PR3-dependent effects on mechanosensation. We decreased S1P levels via injection of the sphingosine kinase inhibitor SKI II to block local production of S1P ([Chiba et al., 2010](#)) or elevated S1P levels via intradermal injection of S1P and measured behaviors 30 min after injection. Decreasing local S1P levels with SKI II significantly reduced mechanical sensitivity ([Figure 2A](#); see [Figure 2—source data 1](#)), comparable to the hyposensitivity phenotype observed in S1PR3 KO mice ([Figure 1A](#)). Again, similar to what was observed in S1PR3 KO animals ([Figure 1E](#)), peripheral blockade of S1P production had no effect on baseline thermal sensitivity ([Figure 1—figure supplement 1C](#)). Surprisingly, injecting exogenous S1P (10 μ M; maximum solubility in saline vehicle) had no effect on mechanical sensitivity ([Figure 2A–B](#)). However, as previously reported ([Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#)), S1P injection triggered S1PR3-dependent thermal hypersensitivity and spontaneous pain ([Figure 2C–D](#)), demonstrating that the lack of effect on mechanical hypersensitivity is not due to problems with S1P delivery or degradation.

Figure 2. Endogenous S1P mediates acute mechanical pain.



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(A) von Frey 50% withdrawal measurements for mice pre- and post-injection of 50 μ M SKI II (N = 8) or 10 μ M S1P (N = 7); p=0.003, 0.604 (two-tailed paired t-tests). (B) von Frey force-response graph for animals injected with 10 μ M S1P or 0.1% MeOH-PBS; $p_{genotype} > 0.05$ (two-way ANOVA; N = 8 mice per group). No

Tukey HSD comparisons at any force between genotypes were significant. **(C)** Intradermal cheek injection of 10 μ M S1P, 2 μ M, 0.2 μ M, and 20 μ L 0.3% methanol PBS (vehicle), with quantification of number of forepaw wipes over the 5 min post-injection interval; $p<0.0001$ (one-way ANOVA; N = 3 mice per condition). Dunnett's multiple comparisons p -values are represented on graph for comparisons made between treated and vehicle groups. **(D)** Radiant heat normalized paw withdrawal latencies 20–30 min post injection of 15 μ L 10 μ M S1P, 0.2 μ M S1P, or 0.3% methanol-PBS vehicle (i.d.) into the hind paw of S1PR3 WT or KO mice; $p=0.0129$ (one-way ANOVA; N = 3–10 mice per condition). Dunnett's multiple comparisons p -values are represented on graph for comparisons made between treated and vehicle groups. **(E)** von Frey 50% withdrawal measurements for mice pre- (baseline) and post-injection of 50 μ M SKI II (N = 14) and 0 (N = 4), 10 (N = 3), 75 (N = 4), or 200 nM S1P (N = 3; one-way ANOVA; $p=0.0001$). Tukey Kramer comparisons are indicated on graph. Error bars represent mean \pm SD.

Figure 2—source data 1. Endogenous S1P mediates acute mechanical pain.

Related to [Figure 2](#).

[eLife-33285-fig2-data1.xlsx](#) (49KB, xlsx)

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These data support a model whereby S1P constitutively activates S1PR3 to set normal mechanical pain thresholds. To further test this model, we asked if the mechanical hyposensitivity elicited after endogenous S1P depletion (via SKI II) could be rescued by local injection of exogenous S1P. Indeed, we found that injection of exogenous S1P reversed SKI II-induced mechanical hyposensitivity in a dose-dependent manner, and observed a maximal effect with 200 nM S1P ([Figure 2E](#)). Although quantification of native S1P levels in skin is inaccurate owing to avid lyase activity ([Shaner et al., 2009](#)), our data establish that baseline S1P levels are sufficient to maximally exert their effect on S1PR3-dependent mechanical pain, such that increased S1P does not evoke mechanical hypersensitivity, but diminished S1P leads to mechanical hyposensitivity. These data show that constitutive activation of S1PR3 by S1P is required for normal mechanosensitivity.

S1PR3 is expressed in A mechanonociceptors and thermal nociceptors

Our behavioral data showing distinct roles for S1PR3 in mechanonociception and thermal hypersensitivity suggest that S1PR3 is expressed in distinct subsets of somatosensory neurons. While a previous study suggested that all somatosensory neurons express S1PR3 ([Camprubí-Robles et al., 2013](#)), single cell RNA seq data suggests *S1pr3* is not

expressed by all DRG neurons ([Usoskin et al., 2015](#)), and no studies have performed quantitative analysis of S1PR3 staining or co-staining to define subpopulations of S1PR3⁺ neurons. We thus set out to characterize the somatosensory neuron subtypes expressing *Slpr3* using *in situ* hybridization (ISH) of wild-type somatosensory ganglia and immunohistochemistry (IHC) in an *Slpr3*^{mCherry/+} reporter mouse ([Sanna et al., 2016](#)).

We first used *in situ* hybridization (ISH) with a specific *Slpr3* probe to examine expression patterns of *Slpr3* ([Figure 3A–B](#); see [Supplementary file 1](#)). In our experiments, 43% of cells from wild-type DRG expressed *Slpr3*. Co-ISH revealed that one population of *Slpr3*⁺ neurons represents A δ mechanonociceptors (AMs). These cells expressed *Scn1a* (39.9% of all *Slpr3*⁺), a gene that encodes the Nav1.1 sodium channel, which mediates mechanical pain in A δ fibers ([Osteen et al., 2016](#)). *Slpr3*⁺ cells also co-expressed *Npy2r* (20.4% of all *Slpr3*⁺), a marker of a subset of mechanonociceptive A fibers ([Arcourt et al., 2017](#)). *Slpr3* was expressed in 70.6% of *Scn1a*⁺ cells and 72% of *Npy2r*⁺ cells, comprising a majority of both of these populations. Interestingly, a subset of cells co-expressed *Slpr3* and the mechanically sensitive channel *Piezo2*, which is expressed by A β , A δ , and C fibers ([Ranade et al., 2014](#)). The remaining *Slpr3*⁺ cells were *Trpv1*⁺ and/or *Trpa1*⁺ C nociceptors (67.1% of all *Slpr3*⁺), which are reported to overlap minimally with the *Scn1a*⁺ and *Npy2r*⁺ populations ([Arcourt et al., 2017](#); [Osteen et al., 2016](#)).

Figure 3. *S1pr3* is expressed in A mechanonociceptors and C thermal nociceptors.

(A) (Top) Representative co-ISH of *S1pr3* (green; left) with *Scn1a*, *Npy2r*, *Piezo2*, and *Trpv1* (magenta; center) in sectioned DRG. Right column: overlay with co-localized regions colored white (10x air objective; scale = 100 μ m). **(B)** Bar chart showing the % of total cells expressing the indicated marker (grey) and the % of total cells co-expressing both marker and *S1pr3* (green). See Table S1 for quantification. **(C)** Representative IHC images of sectioned DRG from *S1pr3*^{mCherry/+} animals stained with anti-DsRed (green, S1PR3) and anti-Peripherin (left, magenta) or anti-NF200 (right, magenta). Arrows indicate co-stained cells. Images were acquired using a 10x air objective (scale = 100 μ m). **(D)** Whole-mount skin IHC confocal images with anti-DsRed antibody (S1PR3, green) and anti-NefH antibody (NF200, magenta) in an *S1pr3*^{mCherry/+} animal (20x water objective; scale = 50 μ m). Arrows indicate co-positive free nerves (left image). Arrowheads indicate NF200- free nerves (left) or S1PR3- circumferential fibers (right image). **(E)** Sectioned skin IHC with anti-DsRed (S1PR3) and anti-NefH (NF200, left, top right) or anti-DsRed (S1PR3) and anti-beta-tubulin III (BTIII, bottom right) antibody (magenta) in *S1pr3*^{mCherry/+} skin (20x air objective; scale = 50 μ m). Arrows indicate co-positive free nerve endings (left), S1PR3-negative lanceolate/ circumferential hair follicle endings (top right, arrow = circumferential, arrowhead = lanceolate), or S1PR3- negative putative Merkel afferent (bottom right). **(F)** (Left) Quantification of sectioned DRG IHC experiments showing % of S1PR3+ cells that co-stained with indicated markers (n > 250 cells per marker). (Right) Quantification of sectioned skin IHC experiments showing % of fibers positive for indicated marker that co-stained with S1PR3 (anti-DsRed; n = 10 images per marker from two animals).

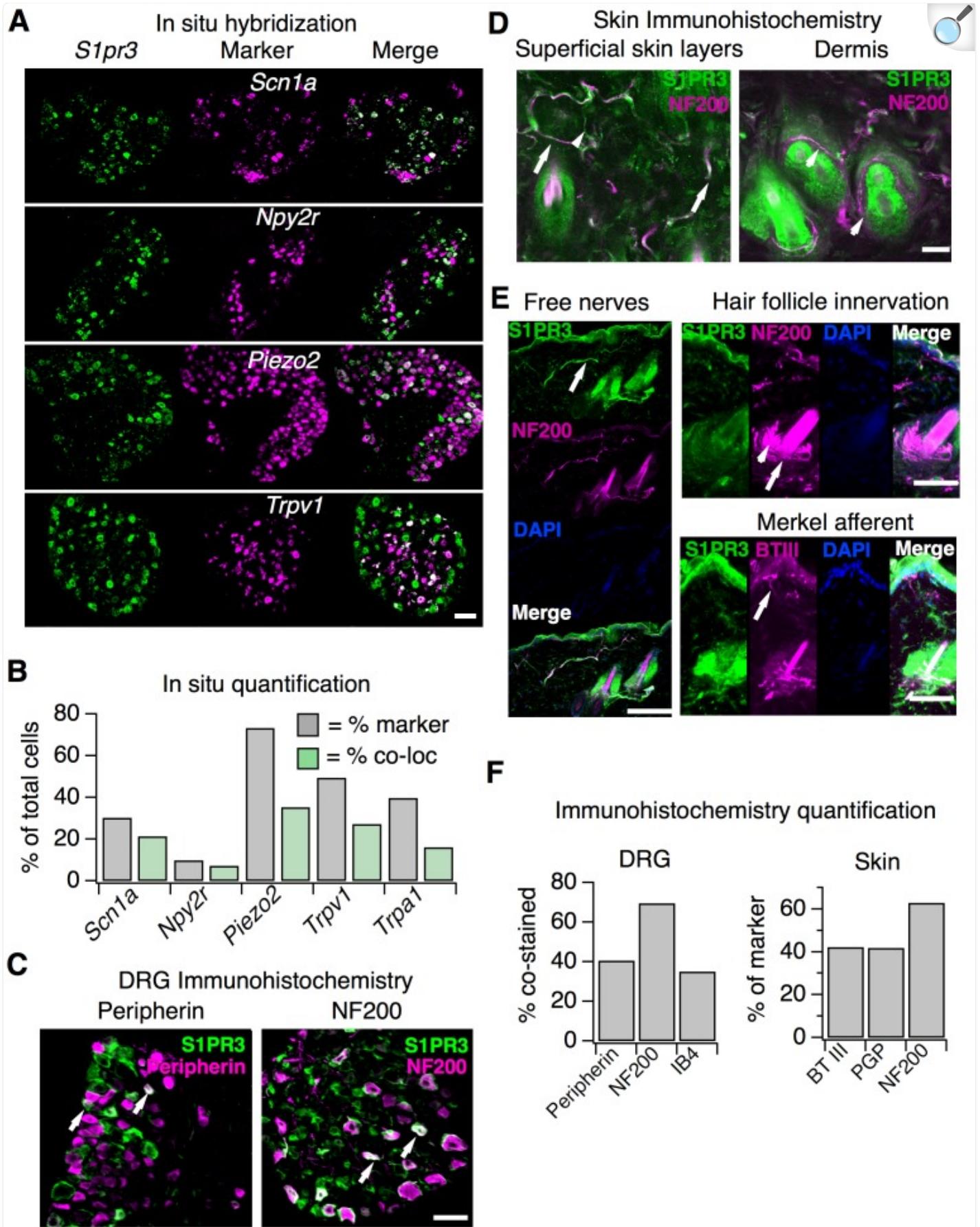
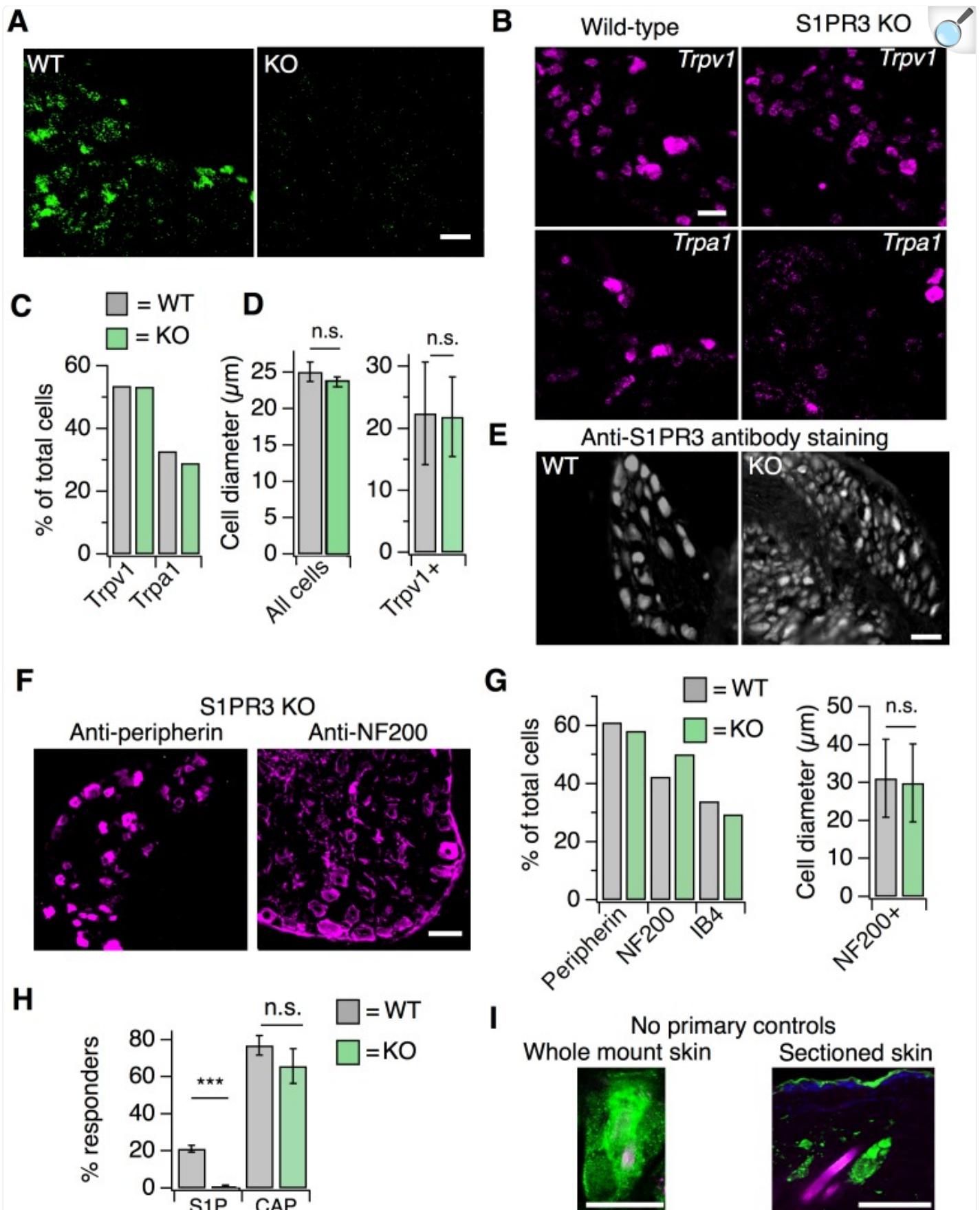


Figure 3—figure supplement 1. S1PR3 KO animals display normal representation of somatosensory neuronal subtypes.



Related to Figure 3. (A) ISH of sectioned adult DRG from WT and S1PR3 KO animals showing specificity of

S1pr3 probes (20x air objective, scale = 50 μm). (B) (Image) Representative ISH *Trpv1* (top) and *Trpa1* (bottom) from sectioned DRG of wild-type (left) and S1PR3 KO animals (right; 20x air objective; scale = 50 μm). (C) % of total cells expressing *Trpv1* and *Trpa1* in sectioned DRG of wild-type and S1PR3 KO animals. (D) (Left) Average diameter \pm SEM of all cells in sectioned DRG from WT and S1PR3 KO animals ($p=0.36$, two-tailed t-test; $n = 437$ and 679 cells from two animals each, respectively). (Right) Average diameter \pm SD of *Trpv1*⁺ cells in WT and S1PR3 KO DRG ($p=0.63$, two-tailed t-test; $n = 127$ and 85 cells, respectively). (E) Representative staining with anti-S1PR3 antibody (1:2000) in sectioned adult DRG from WT and S1PR3 KO animals (10x air objective, scale = 100 μm). (F) Representative IHC images of sectioned DRG from S1PR3 KO animals stained with anti-DsRed (green) and anti-Peripherin (left, magenta) or anti-NF200 (right, magenta). Images were acquired using a 10x air objective (scale = 100 μm). (G) (Left) Quantification of total percentage of cells stained with indicated markers in sectioned DRG from *S1pr3*^{mCherry/+} and S1PR3 KO animals ($n > 250$ cells per condition). (Right) Average diameter of anti-NF200 +cells in *S1pr3*^{mCherry/+} and S1PR3 KO DRG ($p=0.15$, two-tailed t-test; $n = 256$ and 194 cells, respectively). (H) Percent responders to S1P and capsaicin in ratiometric calcium imaging of wild-type and S1PR3 KO cultured DRG and TG neurons; $p<0.0001$ (one-way ANOVA; $N = 2$ DRG and 2 TG preparations of 8 wells each). Sidak's multiple comparisons p -values are represented on graph for comparisons made between genotypes. Error bars represent mean \pm SEM. (I) (Left) No primary control showing robust staining of hair follicles in whole mount skin in contrast to specific neuronal staining shown in [Figure 3D](#). (Right) No primary control showing staining around hair follicles and in epidermis in sectioned skin. Scale = 50 μm (20x water objective).

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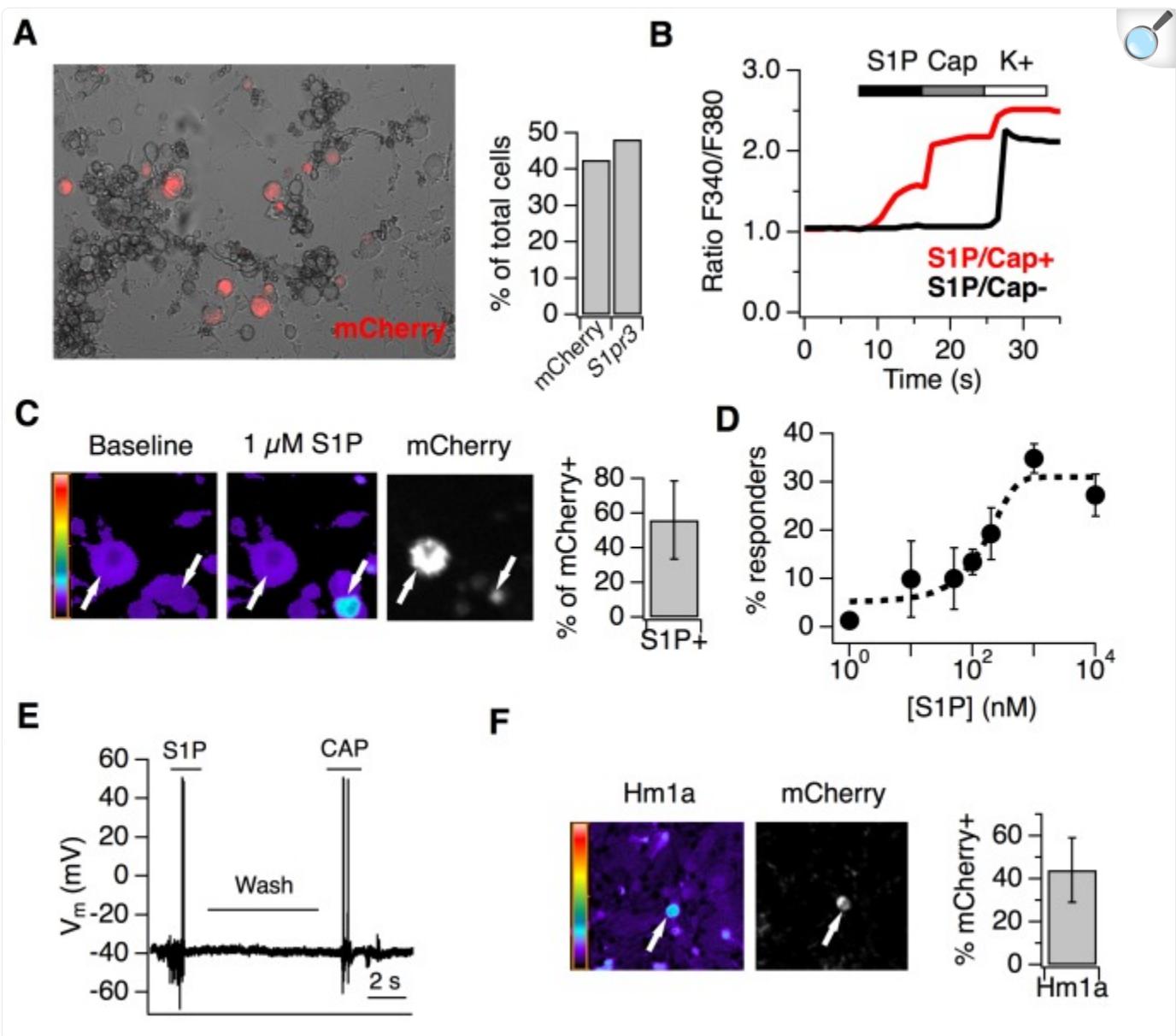
We next used an *S1pr3*^{mCherry/+} reporter mouse, which produces a functional S1PR3-mCherry fusion protein ([Sanna et al., 2016](#)), as an independent strategy to explore S1PR3 expression and localization. This strategy was used because we found that anti-S1PR3 antibodies showed broad immunoreactivity in DRG from mice lacking S1PR3, and so we instead used anti-DsRed antibodies to probe expression of the S1PR3 fusion protein ([Figure 3—figure supplement 1E](#)). We found that 42.4% of S1PR3⁺ cells co-stained with anti-Peripherin, demonstrating that S1PR3 is expressed in a subset of small-diameter neurons. We also observed that 69.5% of S1PR3⁺ cells co-stained with anti-NF200, which marks medium and large-diameter myelinated neurons. Furthermore, we observed that S1PR3⁺ cells were primarily of small to medium diameter (11.3–35.1 μm), whereas all cells in the DRG ranged from 11.3 to 53.9 μm . Overall, these data support the expression of S1PR3 in subsets of small-diameter thermal nociceptors and medium-diameter mechanonociceptors ([Figure 3F](#)). Additionally, no significant differences were observed between WT and S1PR3 KO DRG in number of *Trpa1*⁺, *Trpv1*⁺, Peripherin⁺, NF200⁺, or IB4⁺ cells ([Figure 3—figure supplement 1B–C,F,G](#)). The mean diameters of *Trpv1*⁺ neurons ([Figure 3—figure supplement 1D](#), left), NF200⁺ neurons ([Figure 3—figure supplement 1G](#)), or all neurons ([Figure 3—figure supplement 1D](#), right) in WT versus KO DRG were not significantly different, suggesting no loss of major sensory neuronal subtypes in the S1PR3 KO.

We then visualized S1PR3 expression in nerve fibers that innervate the skin using anti-DsRed antibodies in whole-mount immunohistochemistry (IHC; [Figure 3D](#)). The reporter animals showed no specific antibody staining in epidermal or dermal cells ([Figure 3—figure supplement 1I](#)), and single-cell RNA seq of a diverse array of mouse epidermal and dermal cells corroborates this lack of expression ([Joost et al., 2016](#)). We observed overlap of S1PR3-expressing free nerve endings with NF200⁺ myelinated free nerves and NF200- putative C-fiber endings ([Figure 3F](#)), but did not observe expression of S1PR3 in NF200⁺ circumferential or lanceolate hair follicle receptors, or in putative Merkel afferents ([Figure 3D–E](#)). β -tubulin III, PGP9.5 (pan-neuronal markers), and NF200 staining in S1PR3 KO skin displayed patterns of epidermal and dermal innervation similar to WT skin, suggesting the phenotypes observed in the S1PR3 KO mice are not due to developmental loss of sensory neuronal innervation ($p_{PGP9.5} = 0.443$ ($n = 93$, 38 fibers); $p_{NeFH} = 0.405$ ($n = 61$, 28 fibers); $p_{BTIII} = 0.353$ ($n = 104$, 89 fibers); two-tailed t-tests based on average number of fibers per field of view). These results support expression of S1PR3 in subsets of myelinated A mechanonociceptors and unmyelinated C nociceptors that terminate as free nerve endings.

S1P activates thermal nociceptors but not putative AMs

Live imaging of cultured DRG neurons from adult reporter animals showed expression of S1PR3-mCherry fusion protein in 48.3% of neurons, mirroring our ISH and IHC results ([Figure 4A](#)). To examine the effects of S1P on A mechanonociceptors and C nociceptors, we performed ratiometric calcium imaging and electrophysiology on DRG cultures from reporter mice. Interestingly, only $56.1 \pm 22.4\%$ of mCherry-expressing neurons were activated by 1 μ M S1P (Representative trace in [Figure 4B](#); representative images in [Figure 4C](#)), which our dose-response showed to be the saturating concentration for calcium influx ([Figure 4D](#); $EC_{50} = 155$ nM). All S1P-responsive neurons were also capsaicin-sensitive ($n > 2000$ neurons). And while sensory neurons from S1PR3 KO animals did not respond to S1P, as expected ([Camprubí-Robles et al., 2013](#)), they exhibited capsaicin responses that were not significantly different from WT neurons ([Figure 3—figure supplement 1H](#)). The mean diameter of S1P-responsive mCherry⁺ neurons was 22.4 ± 1.0 μ m, whereas the mean diameter of non-responsive mCherry⁺ neurons was 28.7 ± 3.2 μ m ($p=0.0002$, two-tailed t-test). We also performed whole cell current clamp experiments and, consistent with other studies ([Mair et al., 2011](#); [Zhang et al., 2006](#); [Li et al., 2015](#)), found that S1P evoked action potential firing in capsaicin-sensitive small diameter cells ([Figure 4E](#)). This shows that only the small-diameter, S1PR3⁺ putative nociceptors are excited by S1P. We next asked whether the S1PR3⁺ medium-large diameter neurons represent the mechanonociceptors observed by ISH ([Figure 3A](#)). To this end, we asked whether the spider toxin Hm1a, a selective activator of AM nociceptors ([Osteen et al., 2016](#)), triggers calcium influx in S1PR3-expressing trigeminal neurons. Indeed, we found that $44.2 \pm 15.1\%$ of Hm1a-responsive neurons expressed mCherry ([Figure 4F](#)), consistent with our staining showing expression of *S1pr3* in AM nociceptors and the role of Hm1a-responsive neurons in mediating mechanical pain *in vivo* ([Osteen et al., 2016](#)).

Figure 4. S1P activates thermal nociceptors but not mechanonociceptors.



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(A) (Left) Representative image of mCherry signal in live, cultured adult DRG neurons from one *S1pr3^{mCherry/+}* animal. (Right) Quantification of % of total cells expressing *S1pr3* from DRG ISH and mCherry from dissociated DRG cultures (N = 2 animals each experiment). (B) Representative traces depicting F340/F380 signal from Fura2-AM calcium imaging showing two neurons, one which responded to 1 μ M S1P, 1 μ M Capsaicin, and high K + Ringer's (red) and one which only responded to high K+ (black). (C) (Left) Fura-2 AM calcium imaging before (left) and after (center) addition of 1 μ M S1P in *S1pr3^{mCherry/+}* cultured mouse DRG neurons. Bar indicates fluorescence ratio. Right-hand image indicates mCherry

fluorescence. (Right) % of mCherry neurons that are responsive to 1 μ M S1P in ratiometric calcium imaging ($n > 1000$ cells from 16 imaging wells from three animals). (D) Dose-response curve of mean neuronal calcium responders to varying concentrations of S1P. Concentrations used: 1, 10, 50, 100, 200, 1000, and 10,000 nanomolar ($N = 2$ animals). Error bars represent mean \pm SD. Black dotted line indicates sigmoidal fit for all S1P responders from which EC₅₀ was derived. All S1P responders were also capsaicin-responsive. (E) Current-clamp trace of a single wild-type neuron firing action potentials in response to bath addition of 1 μ M S1P and 1 μ M capsaicin, with Ringer's wash in-between. Four of tenneurons responded to S1P and one of one S1P-responsive also responded to capsaicin. Bar = 2 s. (F) (Left) Fura-2 AM calcium imaging after addition of 500 nM Hm1a in *S1pr3*^{mCherry/+} P0 TG neurons, which were used instead of adult DRG neurons because they respond to Hm1a without prior PGE₂ sensitization. Right-hand image indicates mCherry fluorescence. (Right) % of Hm1a-responsive P0 TG neurons that are mCherry+ ($N = 1$ animal, 1230 total neurons).

S1PR3 modulates KCNQ2/3 channels to regulate AM excitability

We next interrogated the molecular mechanism by which S1P signaling in AM nociceptors may regulate mechanical pain. We performed whole-cell current clamp on the medium-diameter *S1pr3*^{mCherry/+} dissociated DRG neurons (membrane capacitance = 61.05 ± 1.92 pF), which did not display S1P-evoked calcium influx ([Figure 4B–C](#)). In these cells, 1 μ M S1P application did not change membrane potential ([Figure 5—figure supplement 1A](#); see [Figure 5—source data 1](#)) or elicit firing in the absence of current injection ([Figure 5—figure supplement 1A](#); [Figure 5A](#)). However, S1P dramatically lowered the threshold to fire action potentials (rheobase) in an S1PR3-dependent manner ([Figure 5A](#), [Figure 5—figure supplement 1B](#)).

Figure 5. S1PR3 modulates KCNQ2/3 channels to regulate AM excitability.

All experiments were performed in *S1pr3*^{mCherry/+} or ^{-/-} DRG neurons. **(A)** (Left) Example traces of a single mCherry +neuron in whole cell current clamp before and after S1P application. (Right) % change in rheobase after S1P application for *S1pr3*^{mCherry/+} (left, n = 7) and KO (right, n = 12) neurons ($p_{WT,KO} = 0.012, 0.287$; two-tailed paired t-tests). **(B)** % Δ in input resistance after S1P or vehicle application ($p=0.017$; two-tailed paired t-test; n = 4 cells per group). **(C)** The S1P-sensitive current is carried by potassium. The current-voltage relationship was determined by subtraction of the post-S1P current from the pre-S1P current and reverses at -60.125 mV; n = 6 cells. Data were fitted with a Boltzmann equation. Pre- and post-S1P currents were measured at the indicated voltage (-100 mV to $+80$ mV, 20 mV increments) following a $+100$ mV step (100 ms). Current was quantified using the peak absolute value of the slowly-deactivating current 0–10 ms after stepping to indicated voltage. Unless indicated otherwise, all error bars represent mean \pm SEM. **(D)** (Graphic, top) Averaged current traces of a single mCherry+ neuron in whole cell voltage clamp recording comparing tail currents (ΔI_{tail}) pre- and post-S1P using indicated voltage step protocol. (graphic, bottom) Averaged current traces of a single mCherry+ neuron in whole cell voltage clamp recording with XE991 treatment. Holding phase (-40 mV, 150 ms) was truncated in traces. (Left graph) % Δ in outward tail current (average \pm SD after indicated treatments (1 μ M S1P, 3 μ M XE 991, or both) for *S1pr3*^{mCherry/+} medium-diameter neurons; ($p=0.58$; one-way ANOVA; n = 6, 8, 14 cells) using protocol depicted at right. (Right graph) % Δ in inward tail current after indicated treatments (LINO = 100 μ M linopirdine) for *S1pr3*^{mCherry/+} medium-diameter neurons; ($p=0.47$; two-tailed paired t-test; n = 12 cells).

Figure 5—source data 1. S1PR3 modulates KCNQ2/3 channels to regulate AM excitability.

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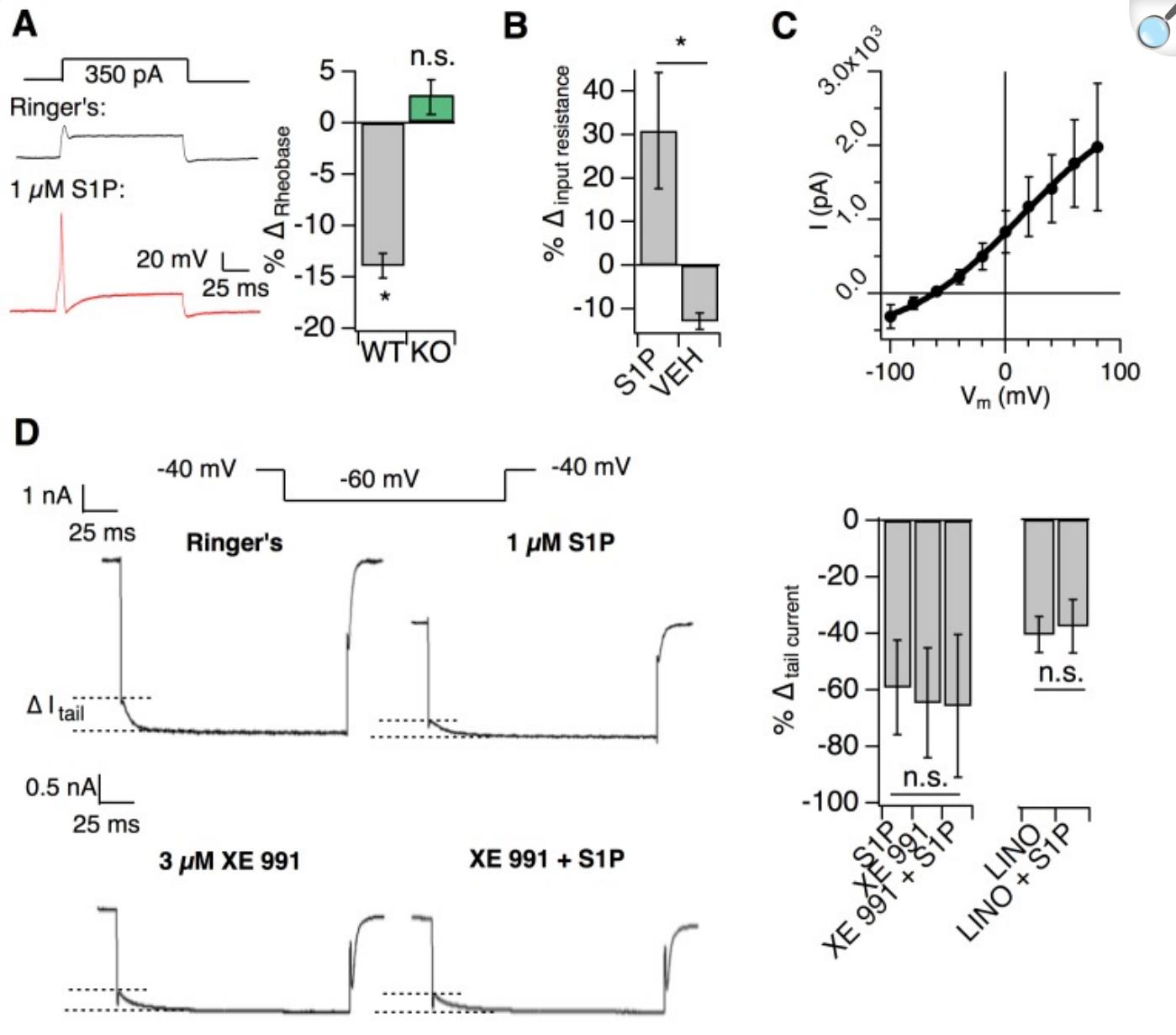
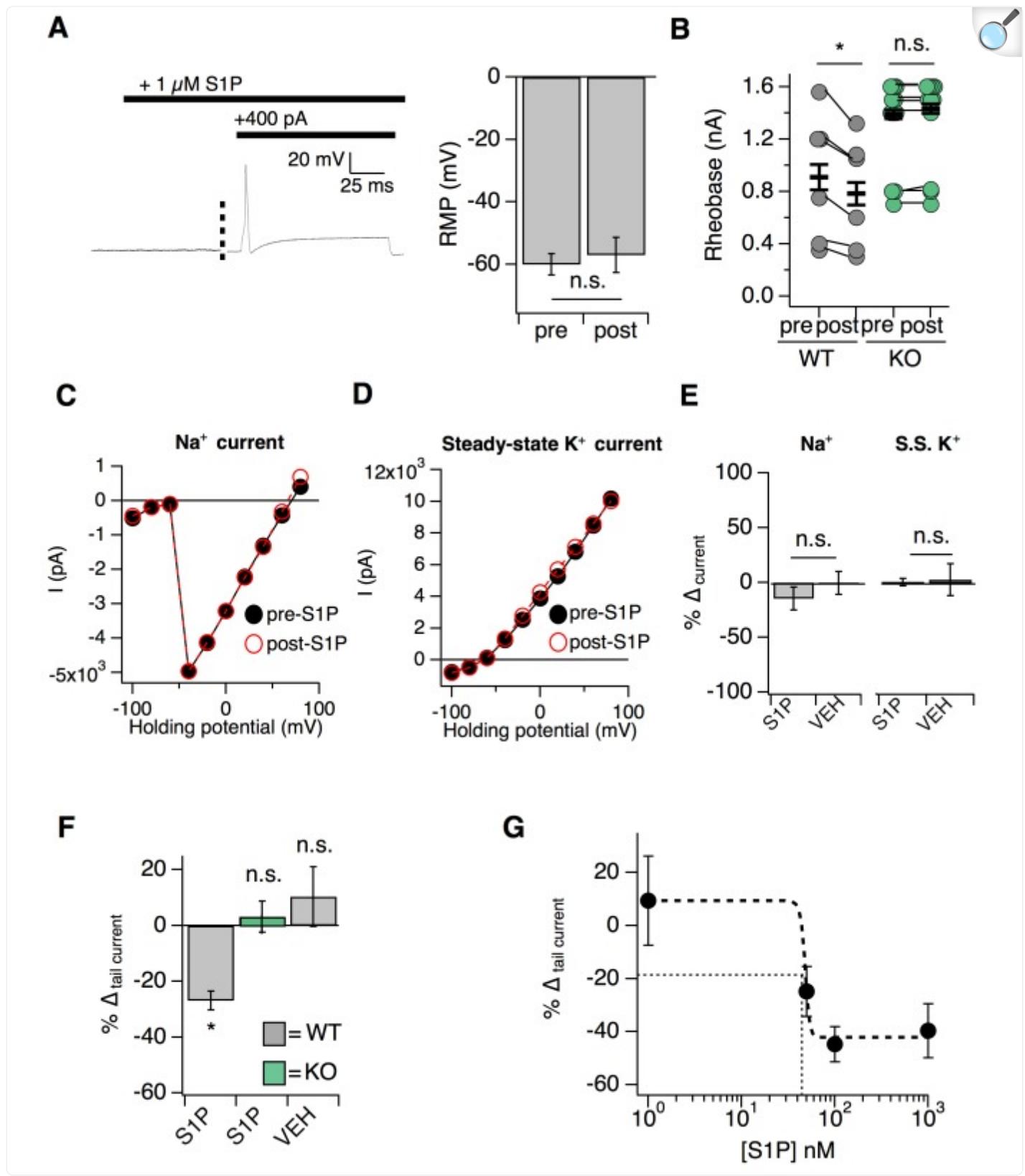


Figure 5—figure supplement 1. S1P selectively modulates potassium tail currents to increase DRG neuron excitability.



Related to [Figure 5](#). **(A)** (Left) Example trace of a single mCherry+ neuron in S1P before and after current injection. (Right) Resting membrane potential (RMP) in millivolts before and after addition of S1P ($p=0.23$; two-tailed paired t-test; $n = 6$ cells). **(B)** Rheobase pre- and post-S1P application in DRG neurons; $p_{WT} = 0.011$; $p_{KO} = 0.28$ (two-tailed paired t-test). Same data are represented in [Figure 5A](#). **(C)** Sodium I-V relationship for a representative *S1pr3*^{mCherry/+} medium-diameter neuron pre- and 5 min post- 1 μ M S1P using voltage step from -100 to +80 mV (150 ms steps, -80 mV holding). **(D)** Steady-state I-V relationship for same neuron. **(E)** (Left) % Δ in peak sodium current (Na^+) after S1P or 1% DMSO vehicle application for medium-diameter mCherry+ neurons; $p=0.39$ (two-tailed paired t-test; $n = 7$ cells per group). (Right) % Δ in peak steady-state current (S.S. K^+) after S1P or 1% DMSO vehicle application for medium-diameter mCherry+ neurons; $p=0.948$ (two-tailed paired t-test; $n = 7$ cells per group). **(F)** % Δ in inward tail current ($\Delta_{I_{tail}}$) after S1P or 1% DMSO vehicle application for *S1pr3*^{mCherry/+} and KO medium-diameter neurons using a pre-pulse stimulation of +80 mV followed by a step to -80 mV, where ($\Delta_{I_{tail}}$) was calculated by subtracting the steady-state current from the absolute peak of the slowly-deactivating current at -80 mV ($p=0.014$; one-way ANOVA; $n = 10, 13, 10$ cells). Tukey Kramer post hoc p -values indicated on graph. **(G)** Dose-response relationship between % Δ in tail current and S1P concentration for 1 nM, 50 nM, 100 nM, and 1 μ M S1P ($n = 7$ cells). EC₅₀ (48.8 nM), marked by thin dotted lines, was estimated from sigmoidal fit (thick dotted line).

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We then set out to determine the mechanism by which S1PR3 activity increases neuronal excitability using whole-cell voltage clamp recording. Previous studies showed that S1P excites capsaicin-sensitive nociceptors by increasing voltage-gated sodium currents and reducing steady-state potassium currents ([Zhang et al., 2006](#); [Li et al., 2015](#)). We found that S1P had no such effects on S1PR3⁺ medium-diameter cells ([Figure 5—figure supplement 1C–E](#)). By contrast, S1P triggered a robust increase in input resistance ([Figure 5B](#)), consistent with the closure of potassium channels. I-V analysis revealed that the current inhibited by S1P application was carried by potassium ([Figure 5C](#)). Additionally, S1P significantly reduced slow, voltage-dependent tail current amplitudes ([Figure 5—figure supplement 1F](#); [Figure 5D](#) (top)) in an S1PR3-dependent manner ([Figure 5—figure supplement 1F](#), center).

As tail currents in A δ neurons are primarily mediated by KCNQ2/3 potassium channels ([Schütze et al., 2016](#); [Passmore et al., 2012](#)), we postulated that S1P may alter tail currents through modulation of these channels. Furthermore, the above properties of the S1P-sensitive current were consistent with the reported electrophysiological properties of KCNQ2/3 channels in DRG neurons ([Schütze et al., 2016](#); [Crozier et al., 2007](#); [Xu et al., 2010](#)). To address whether KCNQ2/3 channels mediated S1P-dependent neuronal excitability, we applied the KCNQ2/3-selective inhibitor XE 991 and found that it completely occluded the effects of S1P on tail current ([Figure 5D](#)). Similar results were observed with the related antagonist, linopirdine ([Figure 5D](#)). These findings are consistent with S1P/S1PR3-dependent inhibition of

KCNQ2/3 in somatosensory neurons.

We also found that the effect of S1P on KCNQ2/3 currents was mediated by low levels of S1P, exhibiting an IC₅₀ of 48 nM with saturation at 100 nM ([Figure 5—figure supplement 1G](#)). While S1P cannot be accurately measured in non-plasma tissues, this is similar to estimated levels of S1P in peripheral tissues ([Schwab et al., 2005](#); [Ramos-Perez et al., 2015](#)), and to levels which rescued mechanosensitivity after local S1P depletion ([Figure 2E](#)). Thus, our in vitro IC₅₀ supports our finding that baseline S1P levels are sufficient to maximally exert their effect on mechanical pain. In summary, our electrophysiological and behavioral observations support a model in which baseline S1P/S1PR3 signaling governs mechanical pain thresholds through modulation of KCNQ2/3 channel activity in AM neurons.

S1PR3 is required for nociceptive responses of high-threshold AM nociceptors

Given the effects of S1P on putative AM neurons in vitro and the selective attenuation of baseline mechanical pain in S1PR3 KO animals, we hypothesized that S1PR3 would play a role in AM afferent function. To test this, we utilized ex vivo skin-nerve recordings to analyze the effects of genetic ablation of S1PR3 on AM afferents, which mediate fast mechanical pain sensation. S1PR3 HET animals were used as littermate controls because no significant differences were observed between S1PR3 WT and S1PR3 HET mice in any behavioral assay ([Figure 1](#)), and because force-response relationships are comparable between S1PR3 HET AM fibers and wild type AM recordings ([Osteen et al., 2016](#); [McIlwrath et al., 2007](#); [Kwan et al., 2009](#); [Smith et al., 2013](#); [Garrison et al., 2012](#)) ([Figure 6—figure supplement 1A](#); see [Figure 6—source data 1](#)). Compared to S1PR3 HET, S1PR3 KO AM nociceptors displayed reduced sensitivity in their force-response relation (slope for HET versus KO: 50 Hz/N versus 35 Hz/N), as well as attenuated firing over the noxious, but not innocuous, range of mechanical stimulation ([Figure 6A](#)). Strikingly, the median von Frey threshold to elicit firing in AM nociceptors was significantly higher in S1PR3 KO animals (3.92 mN) compared to littermate controls (1.56 mN; [Figure 6B](#)). Furthermore, S1PR3 KO AM nociceptors displayed a right-shifted cumulative response curve to force-controlled stimuli (50% effective force for HET versus KO: 33.7 versus 60.0 mN; [Figure 6C](#)), consistent with the mechanonociceptive hyposensitivity observed in vivo. By contrast, neither AM conduction velocities nor the conduction velocity distributions of A β , A δ , and C fibers differed between genotypes ([Figure 6D](#) and [Figure 6—figure supplement 1B](#)).

Figure 6. S1PR3 is required for nociceptive responses of high-threshold AM nociceptors.

(A) (Left) Representative traces of AM fiber activity over time in ex vivo skin-saphenous nerve recording in response to stimulation (128 mN, top) from HET (middle) and KO (bottom) mice. (Right) Mean firing rate of AM fibers in response to force controlled stimulation (4, 8, 16, 32, 64, 128, 256 mN). ** $p=0.001$, *** $p=0.0002$ (two-way ANOVA, Sidak's post-hoc); lines, linear regression (HET: slope = 50 Hz/N, $R^2 = 0.99$; KO: slope = 35 Hz/N, $R^2 = 0.95$). **(B)** von Frey threshold of AM fibers in S1PR3 HET and KO specimens. *** $p<0.0001$ (Mann-Whitney test); lines, median; boxes, 25–75 percentile; whiskers, min-max. **(C)** Cumulative response plot of AM fibers to force controlled stimulation (solid lines); four-parameter logistic fit from which half-maximal force was estimated for each genotype (dotted lines). **(D)** Conduction velocity (CV) of AM fibers in S1PR3 HET and KO mice. $p=0.65$ (two-tailed t-test); $n = 40, 36$ fibers; errors, mean \pm SEM. **(E)** Representative traces and binned instantaneous firing frequencies (IFF; 200 ms bins) of Non-Adapting and Adapting AMs in response to force controlled stimulation (256 mN, top) for S1PR3 HET and KO mice; blue regions, dynamic phase of stimulation (200 ms). **(F)**. Proportion of fibers classified by pattern of mechanically evoked responses to 256-mN stimuli: Non-Responder (HET, 2/40 fibers; KO 5/36), Non-Adapting AM (HET, 18/40; KO, 29/36), Adapting AM (HET, 20/40; KO, 2/36). Non-Responders fired action potentials to large magnitude von Frey monofilaments (<0.5 mm tip diameter), but not controlled mechanical stimulation (256 mN, 2 mm tip diameter). *** $p<0.00001$ (Chi-square test).

Figure 6—source data 1. S1PR3 is required for nociceptive responses of high-threshold AM nociceptors.

Related to [Figure 6](#). Table provides properties of all S1PR3 HET and KO AM fibers that were recorded.

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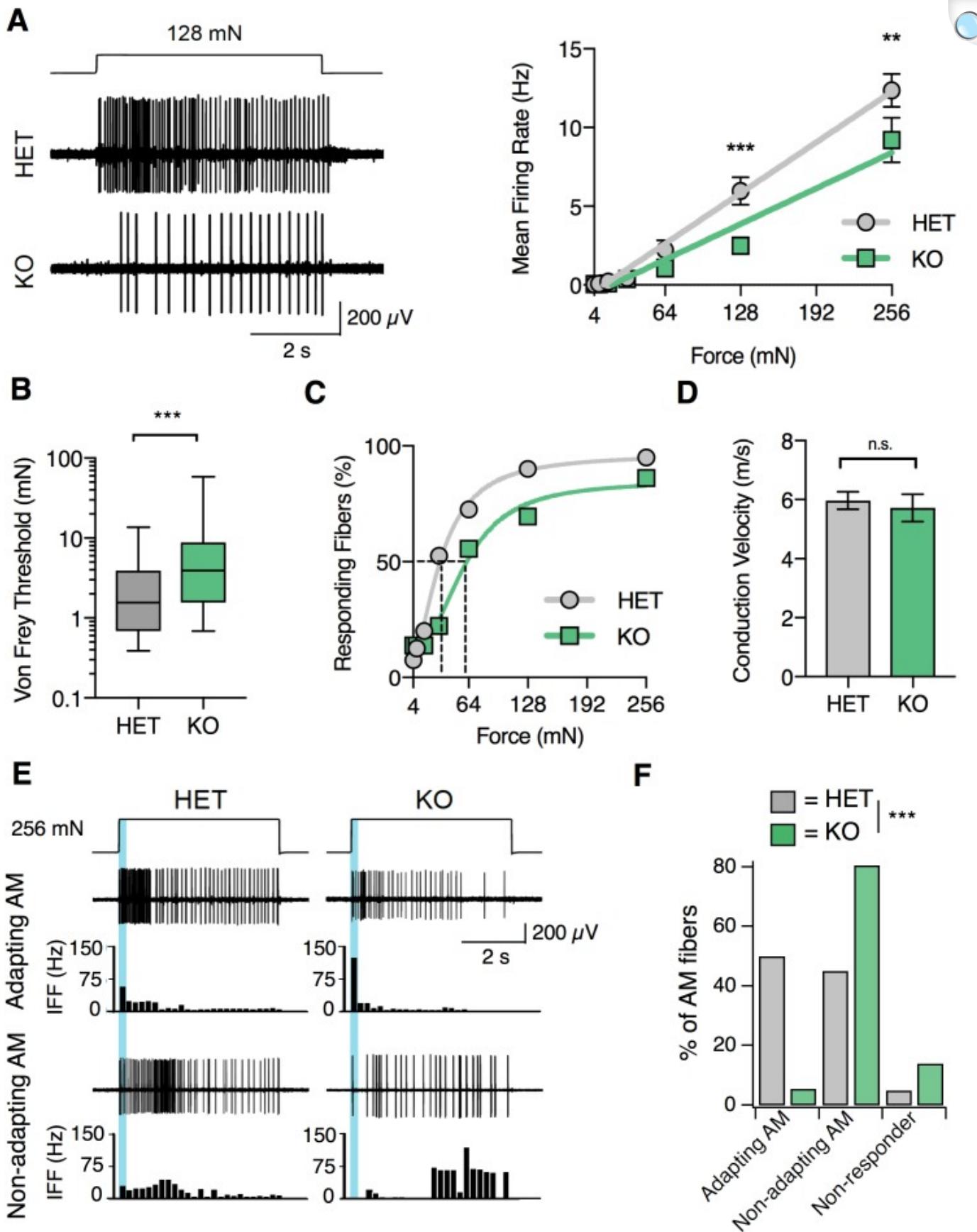
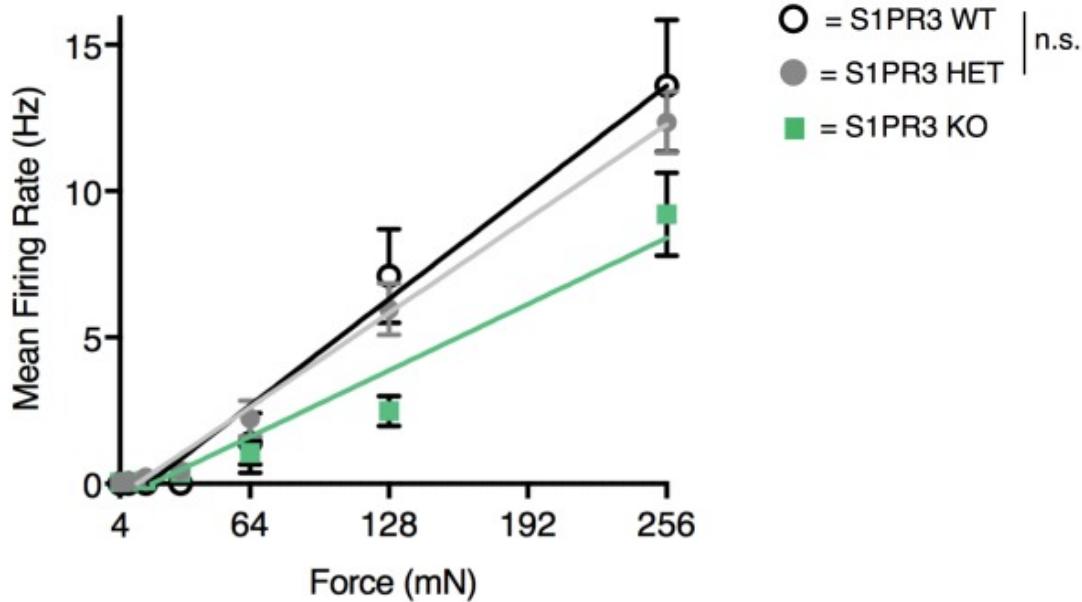
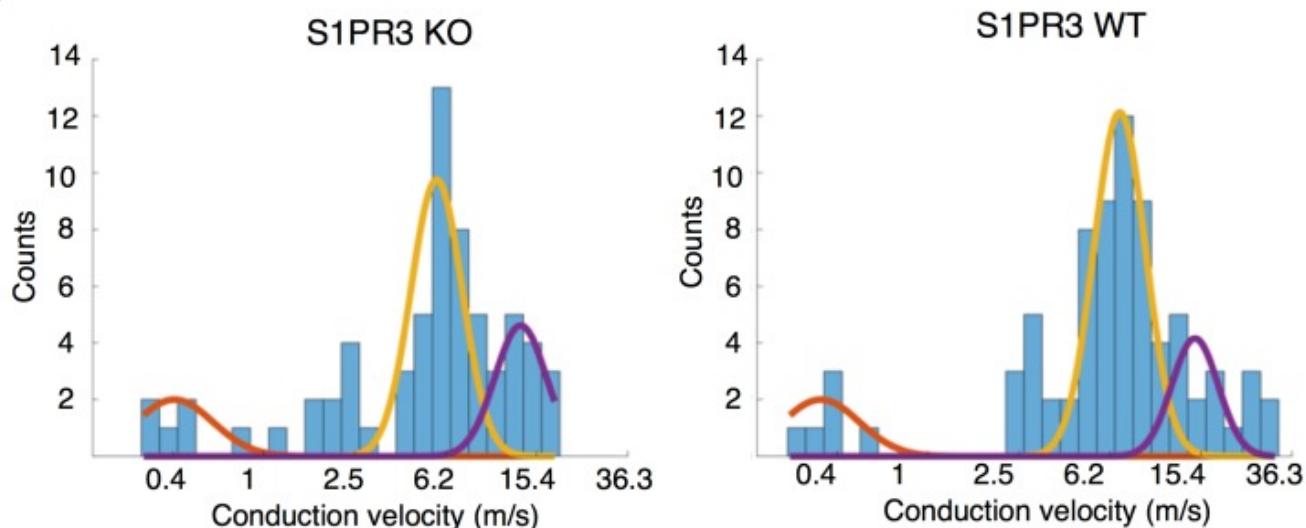
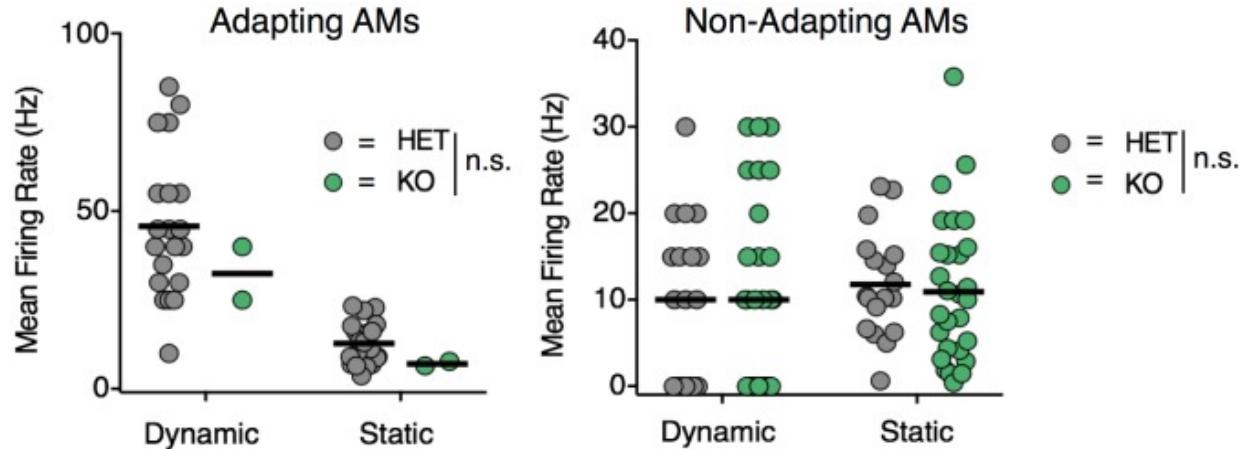


Figure 6—figure supplement 1. S1PR3 HET AM nociceptors display normal nociceptive responses.

A**B****C**

Related to [Figure 6](#). (A) Mean firing rate of AM fibers in response to force controlled stimulation (4, 8, 16, 32, 64, 128, 256 mN) from [Figure 6A](#), with additional data from one wild-type animal ($n_{WT} = 4$ fibers) (WT: slope, 57 Hz/N, R ([Vriens et al., 2011](#)), 0.98; $p=0.90$, two-way ANOVA comparing HET and WT). (B) (Left)

Conduction velocities from teased fibers from 6 S1PR3 KO animals (purple, A β , centroid = 13.1 m/s; yellow, A δ , centroid = 5.7 m/s; orange, C, centroid = 0.4 m/s; R² = 0.69, N = 65 fibers). (Right) Conduction velocities from teased fibers from 1 S1PR3 HET and 6 C57BL/6 WT animals (purple, A β , centroid = 14.5 m/s; yellow, A δ , centroid = 6.8 m/s; orange, C, centroid = 0.3 m/s; R² = 0.82, N = 76 fibers). Three-term Gaussian model. X-axis plotted on a log 1.2 scale. (C) Mean firing rates during dynamic (ramp) and static (hold) stimulation for S1PR3 HET and S1PR3 KO recordings (left, Adapting AMs; right, Non-Adapting AMs; see [Figure 6E–F](#) for experimental details). No significant differences were found between genotypes (p=0.227, 0.490 (two-way ANOVA); bars, means). As shown in [Figure 6F](#), the proportion of Adapting AMs was significantly lower in S1PR3 KO recordings compared with littermate controls.

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A recent study reported that A-nociceptors are composed of two genetically distinct neuronal populations that differ in conduction velocity and in adaptation properties ([Arcourt et al., 2017](#)) ('Adapting AM' versus 'Non-adapting AM'). We next asked whether loss of S1PR3 signaling altered these AM subtypes. Adapting AM fibers responded more vigorously to dynamic (ramp) stimuli than to static (hold) stimuli, and displayed a mean dynamic firing frequency at least twofold greater than their static firing frequency ([Arcourt et al., 2017](#)) ([Figure 6E](#), upper traces). By contrast, Non-adapting AM fibers often showed bursting during static stimulation, which resulted in similar firing rates during dynamic and static stimulation ([Figure 6E](#), lower traces). S1PR3 KO animals displayed a significantly lower proportion of Adapting AM nociceptors compared with littermate controls ([Figure 6F](#)). Additionally, we observed an increase in S1PR3 KO AM fibers that were unresponsive to controlled force stimulation ([Figure 6F](#)). These 'non-responders' only fired action potentials to high-pressure stimuli with a blunt glass probe or to suprathreshold stimulation with von Frey filaments (see Methods). The Non-adapting AMs, and the few remaining mechanosensitive Adapting AMs in the S1PR3 KO displayed similar firing frequencies over both the dynamic and static phases of force application to control fibers ([Figure 6—figure supplement 1C](#)). This suggests that decreased mechanosensitivity of the Adapting AM population accounts for the significant reduction in force-firing relations observed at the population level in S1PR3 KO AMs ([Figure 6A](#)). We conclude that S1PR3 is an essential regulator of both mechanical threshold and sensitivity in a distinct population of AM nociceptors.

S1PR3 is required for inflammatory pain hypersensitivity

Having examined the mechanisms of S1P/S1PR3 signaling in acute mechanonociception, we next sought to evaluate S1P/S1PR3 signaling in pain hypersensitivity. For this purpose, we used an experimental model of inflammatory pain triggered by Complete Freund's Adjuvant (CFA) injection into the hindpaw, which elicits infiltration of immune cells and thermal and mechanical hypersensitivity ([Ghasemlou et al., 2015](#)). While one previous study proposed that S1PR3 promotes injury-evoked heat and mechanical hypersensitivity, they did not measure or compare post-injury mechanical

thresholds to pre-injury baselines for the knockout or control animals ([Camprubí-Robles et al., 2013](#)). Here, we compared development of CFA-evoked hypersensitivity between S1PR3 HET and KO littermates, since no significant behavioral differences were observed between WT and HET animals in CFA experiments ($p_{\text{von Frey}} = 0.12$; $p_{\text{radiant heat}} = 0.12$; two-tailed t -tests). Strikingly, S1PR3 KO mice failed to develop thermal hypersensitivity ([Figure 7A](#); see [Figure 7—source data 1](#)) relative to heterozygous littermates at both 24 and 48 hr post-CFA injection. In stark contrast, S1PR3 KO animals developed robust mechanical hypersensitivity when thresholds were normalized to account for the dramatic baseline differences between knockouts and control animals ([Figure 7B](#)). Our data demonstrate that S1PR3 mediates baseline mechanical sensitivity and is not required for the development of CFA-evoked mechanical hypersensitivity.

Figure 7. S1PR3 is dispensable for development of chronic mechanical hypersensitivity.

(A) Thermal latency before and after CFA treatment (indicated by dotted line); $p_{genotype} = 0.0053$ (two-way ANOVA; N = 5 mice per genotype). Sidak's multiple comparison between genotypes for specific time points indicated on graph. Error bars represent mean \pm SD. **(B)** (Left) Normalized 50% withdrawal threshold before and after CFA treatment (indicated by dotted line); $p(\text{genotype}) < 0.001$ (two-way ANOVA). (Right) 50% withdrawal thresholds for same experiment ($p(\text{genotype}) = 0.1634$; two-way ANOVA). **(C)** (Left) Thermal latency assessed before ('Baseline') and 24 hr post CFA injection with either vehicle (CFA + VEH) or TY 52156 (CFA + TY) acutely administered; $p < 0.0001$ (one-way ANOVA N = 5 mice per treatment). (Right) Thermal latency assessed before and after CFA injection with either vehicle (CFA + VEH) or SKI II (CFA + SKI II) acutely administered on Day 1; $p < 0.0001$ (one-way ANOVA; N = 5–7 mice per treatment). Dunnett's test comparisons to baseline are indicated on graph. Error bars represent mean \pm SD. **(D)** (Left) 50% withdrawal threshold assessed before and 24 hr post CFA injection with either vehicle (CFA + VEH) or TY 52156 (CFA + TY) acutely administered on Day 1; $p < 0.0001$ (one-way ANOVA; N = 5 mice per treatment). Dunnett's test comparisons to baseline are indicated on graph. (Right) 50% withdrawal threshold assessed before and 24 hr post CFA injection with either vehicle (CFA + VEH) or SKI II (CFA + SKI II) acutely administered; p -values indicated on graph (two-tailed unpaired t-test; N = 5 mice per group).

Figure 7—source data 1. S1PR3 is dispensable for development of chronic mechanical hypersensitivity.

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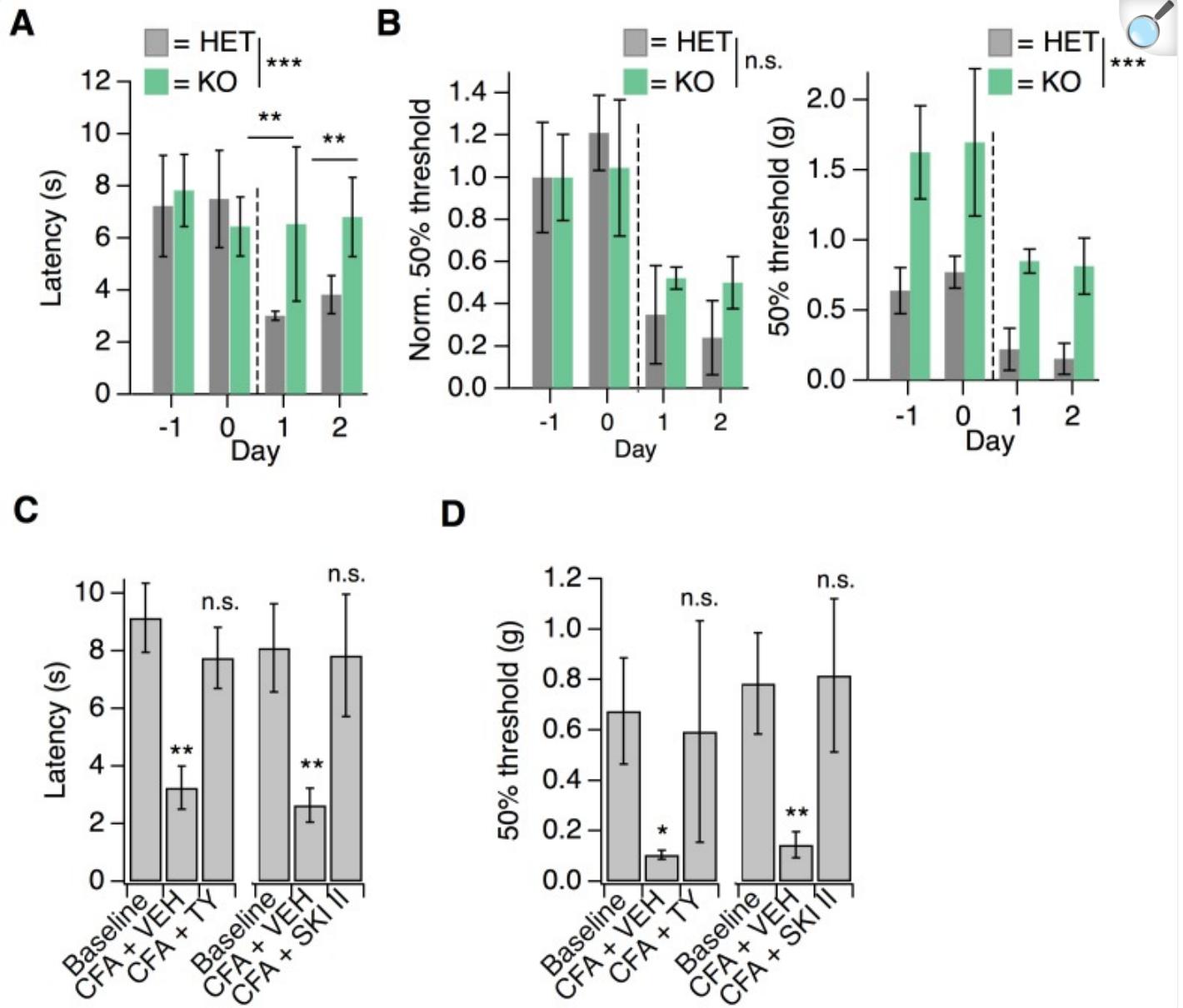
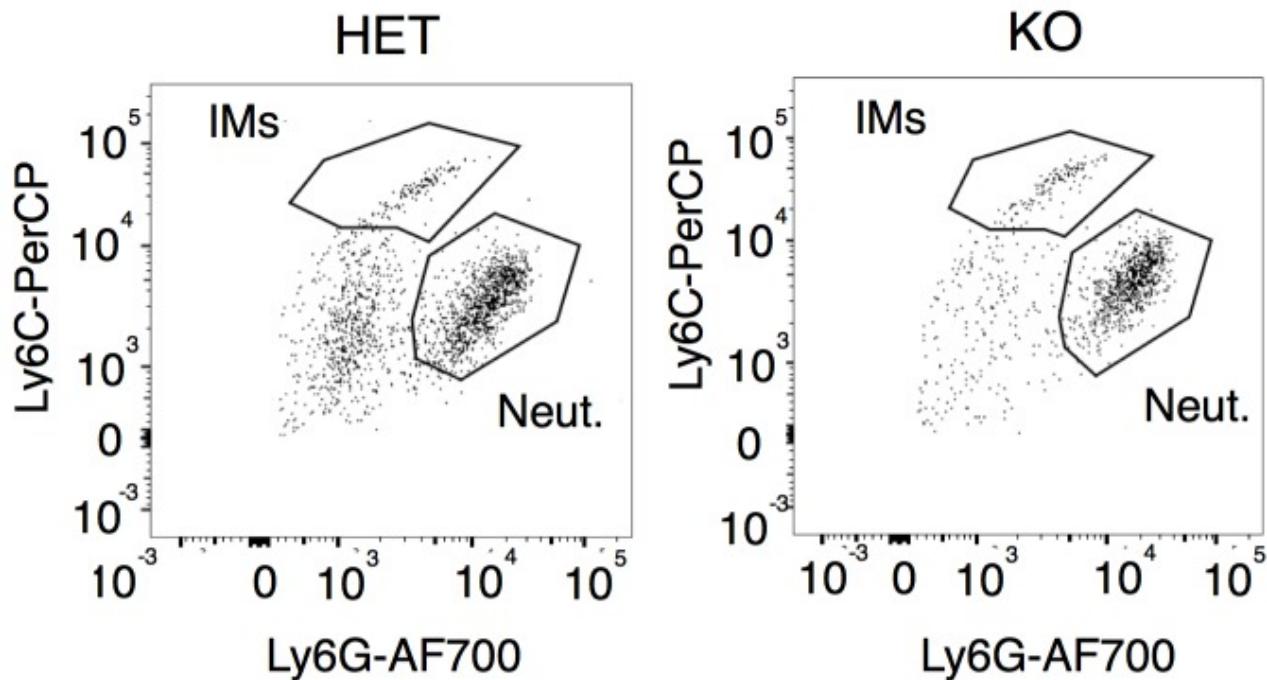
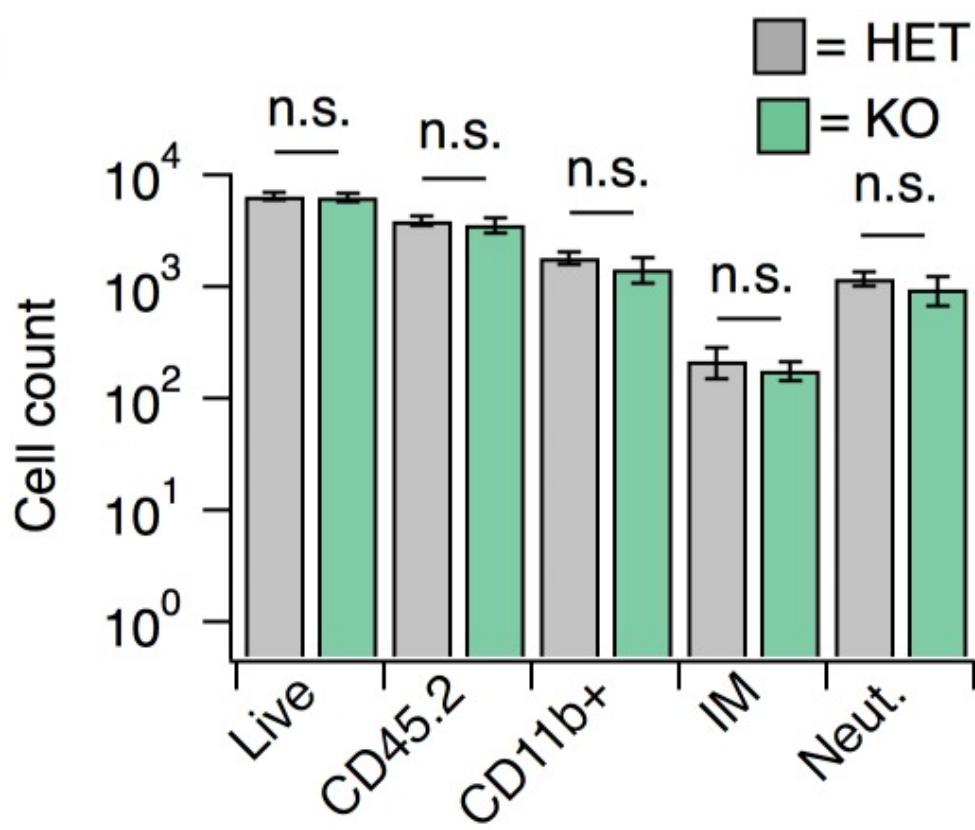


Figure 7—figure supplement 1. S1PR3 KO animals display normal CFA-evoked immune cell recruitment.

A



B



Related to [Figure 7](#). **(A)** Recruitment of neutrophils (Neut.) or inflammatory monocytes (IMs) to hindpaw skin 24 hr post-CFA administration in S1PR3 HET and KO mice, as a dot plot of CD11b⁺ cells plotting Ly6G fluorescence intensity vs. Ly6C intensity (AFU). Boxes are for illustrative purposes. **(B)** Recruitment of immune cells, including neutrophils (Neut.) and inflammatory monocytes (IMs) to hindpaw skin 24 hr post-CFA administration in HET and KO mice, displayed as total number of cells; N = 8 mice per genotype. Sidak's multiple comparisons were made between HET and KO for each cell type.

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Influx of myeloid lineage (Cd11b⁺/Ly6G⁻) cells is required for the development of mechanical hypersensitivity in the CFA model ([Ghasemlou et al., 2015](#)). In the immune system, S1P signaling via S1PR1 plays a key role in immune cell migration ([Matloubian et al., 2004](#)). Consistent with the development of mechanical hypersensitivity, flow cytometry experiments showed robust infiltration of immune cells into hindpaw skin from both S1PR3 KO and littermate controls ([Figure 7—figure supplement 1A–B](#)). These data suggest that the phenotypes observed in S1PR3 KO mice cannot be attributed to compromised immune cell infiltration.

Next, we tested whether active S1P/S1PR3 signaling was required to maintain CFA-evoked thermal hypersensitivity using pharmacology. Acute blockade of S1P production with SKI II or S1PR3 with TY also reversed CFA heat hypersensitivity ([Figure 7C](#)), demonstrating that peripheral S1P actively signals via S1PR3 to promote CFA-evoked heat hypersensitivity. Furthermore, acute S1P/S1PR3 blockade with SKI II or TY elevated mechanical thresholds to pre-CFA, baseline levels ([Figure 7D](#)) showing that S1PR3 tunes mechanical pain under normal and inflammatory conditions. These results are consistent with the distinct roles for AM and C nociceptors in mechanical pain. Under normal conditions, AM nociceptors set mechanical pain thresholds ([Osteen et al., 2016](#); [Abrahamsen et al., 2008](#)). By contrast, under inflammatory conditions, the combined activity of both non-sensitized AM fibers and sensitized C fibers determines overall post-inflammatory mechanical thresholds ([Lennertz et al., 2012](#); [Abrahamsen et al., 2008](#)). Consistent with this model, acute blockade of S1P production or S1PR3 activity under normal conditions induces mechanical hyposensitivity and under inflammatory conditions returns mechanical sensitivity to normal levels.

Discussion

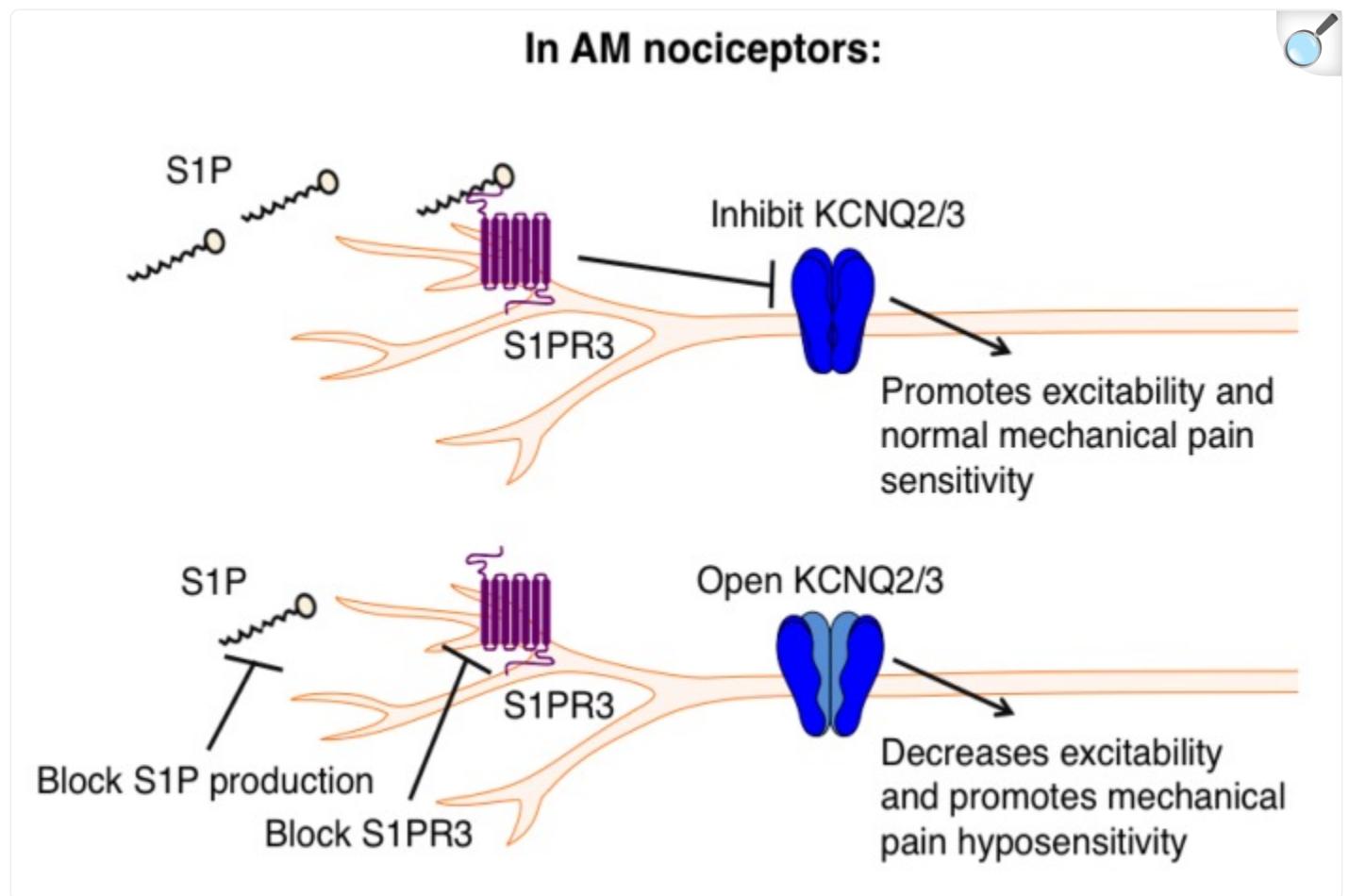
We now show that S1P signaling via S1PR3 is a key pathway that tunes mechanical pain sensitivity. Overall, our data reveal two new key findings. First, S1P/S1PR3 sets baseline mechanical pain thresholds. Depletion of baseline, endogenous S1P induces mechanical hyposensitivity and nanomolar levels of exogenous S1P are sufficient to restore normal mechanical pain sensitivity after depletion. Second, elevated micromolar S1P levels, such as those produced during inflammation or disease, promote thermal, but not mechanical hypersensitivity. The effects of S1P on acute

mechanical pain and thermal hypersensitivity are completely lost in S1PR3 knockout animals, which are otherwise normal with respect to other somatosensory behaviors.

What is the local source of S1P in the skin that constitutively modulates mechanical pain? Even in the mature field of S1P signaling in the vascular and immune systems, the cellular source of S1P, while an intriguing question, remains unclear. All cells in the body, including somatosensory neurons, immune cells, and skin cells, express sphingosine kinases 1 and 2 which are essential for S1P production ([Chalfant and Spiegel, 2005](#)). Deletion of both kinases is lethal and attempts to conditionally knockout these kinases fail to completely eliminate S1P in tissues ([Pappu et al., 2007](#)). While RNA seq data suggests that somatosensory neurons contain all of the enzymatic machinery required to produce and export local S1P ([Usoskin et al., 2015](#); [Morita et al., 2015](#)), future work will be needed to identify the key cell types that are important for maintaining baseline S1P levels in the skin to regulate mechanical sensitivity and for increasing S1P under inflammatory/injury conditions to promote pain hypersensitivity.

Recent studies have identified distinct populations of AM nociceptors that are required for mechanical pain ([Arcourt et al., 2017](#); [Ghitani et al., 2017](#)). Likewise, it was discovered that a subset of somatostatin-expressing spinal interneurons is required for mechanical pain transduction ([Duan et al., 2014](#)). Although these papers delineate the cells and circuitry of mechanical pain, the molecular underpinnings of mechanonociception in the periphery are poorly understood. While the identity of the transduction channel(s) in AM nociceptors remains enigmatic, understanding molecular mechanisms that regulate excitability will no doubt provide key insights into the function and specialization of the diverse subtypes of mechanosensitive nerve fibers. For example, although *Piezo2*-hypomorphic animals exhibit normal mechanical pain behaviors ([Ranade et al., 2014](#)), ex vivo skin-nerve recordings show that their AM nociceptors display decreased force-responsiveness ([Ranade et al., 2014](#)), and a recent study found that subpopulations of sensory neurons express different splice variants of *Piezo2* that exhibit different force sensitivities ([Szczt et al., 2017](#)). These studies suggest that mechanosensitive neurons exhibit functional specialization on multiple levels. Our study demonstrates that S1PR3 is indispensable for normal function of AM nociceptors, including the adapting AM population, recently discussed in Arcourt et al., that innervates the epidermis and encodes noxious touch ([Arcourt et al., 2017](#)). We show that S1PR3 signaling modulates KCNQ2/3 channels to regulate excitability of these A mechanonociceptors ([Figure 8](#)).

Figure 8. Proposed model illustrating a key role for S1PR3 in regulating mechanical pain in AM nociceptors.



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(Top) S1P promotes activation of S1PR3, which leads to inhibition of KCNQ2/3 currents and promotes normal mechanical pain sensitivity. (Bottom) Diminished S1P or S1PR3 antagonism alleviates inhibition of KCNQ2/3, leading to mechanical pain hyposensitivity.

GPCR-mediated inhibition of KCNQ2/3 potassium channels is a well-known mechanism by which neuronal excitability is regulated ([Passmore et al., 2003](#)). Other studies have shown that KCNQ channels mediate excitability of A δ fibers ([Schütze et al., 2016](#); [Passmore et al., 2012](#)) and are required for normal mechanonociceptive responses in dorsal horn neurons receiving A δ input ([Passmore et al., 2012](#)), and that opening KCNQ2/3 channels directly with retigabine alleviates pain in vivo ([Xu et al., 2010](#); [Hayashi et al., 2014](#); [Blackburn-Munro and Jensen, 2003](#)). Our results not only complement previous work implicating KCNQ2/3 channels in pain, but also define the upstream mechanisms that

promote the regulation of KCNQ2/3 channels to tune mechanical pain thresholds. Our data thus highlight S1PR3 as a novel and attractive target for the treatment of mechanical pain and describe a new signaling pathway regulating AM nociceptor excitability.

Interestingly, the neurons that innervate the ultra-sensitive tactile organ of the star-nosed mole are highly enriched in transcripts for S1PR3 and KCNQ channels, as well as for a variety of other potassium channels ([Gerhold et al., 2013](#)). While it is difficult to directly examine the physiological basis for heightened mechanosensitivity in the star-nosed mole, S1PR3-dependent modulation of KCNQ may represent an important mechanism underlying the high tactile sensitivity of the star organ. Moreover, the link between *S1pr3* and *Kcnq2/3* is echoed in single-cell RNA seq datasets from mouse DRG neurons, which show co-expression of *S1pr3* and *Kcnq2/3* in a subset of myelinated mechanoreceptors ([Usoskin et al., 2015](#)). These cells are distinct from the *S1pr3/Trpv1* subset that mediates S1P-evoked acute pain and heat hypersensitivity. In addition to being transcriptionally distinct, we show that the mechanisms underlying S1P's activities in these cells are functionally distinct. Finally, *S1pr3* and *Kcnq2/3* are highly expressed in human sensory ganglia ([Ray et al., 2018](#); [Flegel et al., 2015](#)) and recordings from human stem cell-derived sensory neurons show that KCNQ2/3 channels play a key role in mediating their excitability ([Young et al., 2014](#)). Thus S1PR3 signaling may represent a new target for modulating mechanical pain.

Previous studies of S1P signaling in DRG neurons focused on S1P-evoked excitation of small diameter and/or capsaicin-sensitive neurons, and pain behaviors triggered by elevated S1P. While our new data affirms the effects S1P in thermal nociceptors observed by others, our manuscript highlights a novel effect of baseline levels of S1P in modulation of rheobase and KCNQ2/3 currents in mechanonociceptors. We are also the first to examine the role of S1PR3 in a variety of somatosensory behaviors under normal conditions (non-injury, no algogen injection), and to demonstrate a key role for S1PR3 in mechanonociception. We also go beyond previous studies ([Mair et al., 2011](#); [Camprubí-Robles et al., 2013](#); [Finley et al., 2013](#); [Weth et al., 2015](#)) in showing that elevated S1P selectively promotes thermal and not mechanical hypersensitivity. Others have shown that there are distinct cells and molecular pathways that trigger thermal versus mechanical hypersensitivity. For example, thermal hypersensitivity in the CFA model is dependent on TRPV1 ion channels ([Caterina et al., 2000](#)) and independent of immune cell infiltration ([Ghasemlou et al., 2015](#)). By contrast, infiltration of a subset of myeloid immune cells is required for mechanical hypersensitivity in an inflammatory pain model ([Ghasemlou et al., 2015](#)). Here we show that S1P via S1PR3 signaling is a key component of the inflammatory soup that triggers thermal hypersensitivity in the CFA model. Our observation that S1PR3 KO animals display normal immune cell infiltration and develop mechanical hypersensitivity after CFA is consistent with these previous studies showing distinct mechanisms of inflammatory thermal and mechanical hypersensitivity.

Outside of the nervous system, S1P signaling via S1PR1 allows for the continuous circulation of lymphocytes between blood, lymph, and peripheral tissues ([Matloubian et al., 2004](#)). Our findings that S1P plays a key role in noxious mechanosensation are in line with recent studies showing that sensory neurons co-opt classical immune pathways to drive chronic itch or pain ([Oetjen et al., 2017](#); [Pinho-Ribeiro et al., 2017](#)). What distinguishes this study from the others

is that S1P signaling is critical for acute mechanical pain, even in the absence of inflammation or exogenously elevated S1P. In the immune system, disruptions in S1P levels or S1PR1 signaling result in significant immune dysfunction and disease ([Donoviel et al., 2015](#); [Gräler and Goetzl, 2004](#); [Olivera et al., 2013](#)). Accordingly, in the somatosensory system, excessive, high levels of S1P (micromolar), such as those present in inflammation, evokes thermal pain and sensitization. Intermediate, baseline levels (nanomolar) regulate AM excitability and are required for normal mechanical pain sensation. By contrast, lowering S1P levels reduces mechanical pain sensation, while sparing innocuous touch sensation.

We propose that S1PR3 signaling may contribute to a variety of inflammatory diseases. S1P has been linked to a wide range of human inflammatory disorders ([Allende et al., 2011](#); [Kunkel et al., 2013](#); [Roviezzo et al., 2015](#); [Liang et al., 2013](#); [Rivera et al., 2008](#); [Myśliwiec et al., 2017](#); [Checa et al., 2015](#)). Canonically, S1P signaling via S1PR1 is thought to promote inflammation via the immune system ([Kunkel et al., 2013](#)), however, we propose that S1P signaling via S1PR3 in neurons may also contribute to inflammatory disease. Indeed, fingolimod, a non-selective S1PR modulator, is prescribed as an immunosuppressant for multiple sclerosis treatment ([Brinkmann et al., 2010](#)), but the possibility that some of its therapeutic effects may also be mediated via the nervous system has not been fully explored. Likewise, one study found that intrathecal fingolimod reduces bone cancer pain ([Grenald et al., 2017](#)), and while analgesia was attributed to effects on S1PR1 in glia, some of the benefits may be due to S1PR3 signaling in DRG neurons. S1PR3 antagonism may also be useful in the treatment of inflammatory pain due to its selective dampening of acute mechanical pain and inflammatory thermal hypersensitivity, while preserving innocuous touch and normal thermal sensitivity. S1PR3 inhibitors may also be beneficial for treating other inflammatory disorders where S1PR3-expressing somatosensory neurons have been shown to contribute to neurogenic inflammation, such as asthma ([Tränkner et al., 2014](#)). Our study demonstrates a crucial role for S1P signaling in the peripheral nervous system and highlights the potential of S1PR3 as a target for future pain therapies.

Materials and methods

Key resources table.

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
strain, strain background (C57BL/6J)	C57BL/6J; WT; wild-type	The Jackson Laboratory	Jackson Stock #: 000664; RRID: IMSR_JAX:000664	
		MMRRC Repository; https://www.ncbi.nlm.nih.gov/pubmed/15138255 ;	B6.129S6-S1pr3tm1Rlp/Mmnc; MMRRC Stock #:	
strain, strain background (B6.129S6- S1pr3tm1Rlp/Mmnc)	S1PR3 KO; S1pr3/-	PMID: 15138255	012038-UNC; RRID: MMRRC_012038-UNC	
strain, strain background (B6.Cg- S1pr3tm1.1Hrose/J)	<i>Slpr3-mCherry</i> ; <i>Slpr3mcherry</i> /+	The Jackson Laboratory	B6.Cg-S1pr3tm1.1Hrose/J; Jackson Stock #: 028624; RRID: IMSR_JAX:028624	
antibody (Living Colors DsRed)				
Rabbit Polyclonal Antibody)	Rabbit anti-DsRed	Clontech	RRID: AB_10013483 ; Cat # 632496	
antibody (Chicken polyclonal to Neurofilament heavy polypeptide)	Chicken anti-NefH	Abcam	RRID: AB_304560 ; Cat # ab4680	
antibody (Chicken polyclonal to beta III Tubulin)	Chicken anti- β -tubulin III	Abcam	RRID: AB_10899689 ; Cat # ab107216	
antibody (Mouse monoclonal [13C4/I3 C4] to PGP9.5)	Mouse anti-PGP9.5	Abcam	RRID: AB_306343 ; Cat # ab8189	
antibody (Rabbit polyclonal to EDG3)	Rabbit anti-S1PR3	Abcam	RRID: AB_732070 ; Cat # ab38324	

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
antibody (Mouse monoclonal to NF200)	Mouse anti-NF200	Sigma-Aldrich	RRID: AB_260781 ; Cat # N5389	
antibody (Chicken polyclonal to Peripherin)	Chicken anti-Peripherin	Abcam	RRID: AB_777207 ; Cat # ab39374	
antibody (Goat Anti-Mouse IgG H and L Alexa Fluor 488)	Goat anti-Mouse Alexa 488	Abcam	RRID: AB_2688012 ; Cat # ab150117	
antibody (Goat anti-Chicken IgY (H + L) Secondary Antibody, Alexa Fluor 488)	Goat anti-Chicken Alexa 488	ThermoFisher Scientific	RRID: AB_2534096 ; Cat # A-11039	
antibody (Goat anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor 594)	Goat anti-Rabbit Alexa 594	Invitrogen	RRID: AB_2556545 ; Cat # R37117	
sequence-based reagent	<i>Slpr3</i> Type I Probe	ThermoFisher Scientific; Affymetrix	Assay ID: VB1-19668-VC	
sequence-based reagent	<i>Scn1a</i> Type 6 Probe	ThermoFisher Scientific; Affymetrix	Assay ID: VB6-18173-VC	
sequence-based reagent	<i>Npy2r</i> Type 6 Probe	ThermoFisher Scientific; Affymetrix	Assay ID: VB6-3197254-VC	
sequence-based reagent	<i>Piezo2</i> Type 6 Probe	ThermoFisher Scientific; Affymetrix	Assay ID: VB6-18046-VC	

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
sequence-based reagent	<i>Trpv1</i> Type 6 Probe	ThermoFisher Scientific; Affymetrix	Assay ID: VB6-18246-VC	
sequence-based reagent	<i>Trpa1</i> Type 6 Probe	ThermoFisher Scientific; Affymetrix	Assay ID: VB6-16610-VC	
commercial assay or kit (ViewRNA ISH Tissue Assay Kit (2-plex))	ViewRNA ISH Tissue Assay Kit	ThermoFisher Scientific; Affymetrix	Cat # QVT0012	
chemical compound, drug (Sphingosine-1-phosphate)	Sphingosine 1-phosphate; S1P	Tocris Bioscience; Avanti Polar Lipids	CAS 26993-30-6; Cat # 1370; Cat # 860641	
chemical compound, drug (TY 521256)	TY 52156	Tocris Bioscience	CAS 934369-14-9; Cat # 5328	
chemical compound, drug (SKI II)	SKI II	Tocris Bioscience	CAS 312636-16-1; Cat # 2097	
chemical compound, drug (Histamine dihydrochloride)	Histamine	Sigma-Aldrich	CAS 56-92-8; Cat # H7250	
chemical compound, drug (Chloroquine diphosphate)	Chloroquine	Sigma-Aldrich	CAS 50-63-5; Cat # C6628	
chemical compound, drug (E-Capsaicin)	Capsaicin	Tocris Bioscience	CAS 404-86-4; Cat # 0462	
chemical compound, drug (Dimethyl sulfoxide)	DMSO	Sigma-Aldrich	Cat # 8418–100 mL	
chemical compound, drug (Methanol)	Methanol	Sigma-Aldrich	CAS 67-56-1; Cat # 34860	
chemical compound, drug (Linopirdine)	Linopirdine	Tocris Bioscience	CAS 113168-57-3; Cat # 1999	

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
dihydrochloride)				
chemical compound, drug (XE 991 dihydrochloride)	XE 991	Tocris Bioscience	CAS 122955-13-9; Cat # 2000	
chemical compound, drug (W146)	W146	Tocris Bioscience	CAS 909725-61-7; Cat # 3602	
chemical compound, drug (Freund's Adjuvant, Complete)	Complete Freund's Adjuvant; CFA	Sigma-Aldrich	Cat # F5881	
chemical compound, drug (Formaldehyde, 16%, methanol free, Ultra Pure)	Paraformaldehyde; PFA	Polysciences, Inc.	Cat # 18814-10	
chemical compound, drug (Tissue Tek Optimal cutting temperature compound (OCT))	OCT	Sakura Finetek USA	Cat # 4583	
chemical compound, drug (Triton X-100 solution)	Triton X-100	BioUltra	CAS 9002-93-1; Cat # 93443	
chemical compound, drug (Phosphate- buffered saline (PBS), pH 7.4)	PBS	Gibco	Cat # 10010023	
chemical compound, drug (Benzyl benzoate)	Benzyl benzoate	Sigma-Aldrich	CAS 120-51-4; Cat # B6630	
chemical compound, drug (Benzyl alcohol)	Benzyl alcohol	Sigma-Aldrich	CAS 100-51-6; Cat # 305197	
chemical compound,	Sucrose	Sigma-Aldrich	CAS 57-50-1; Cat # S0389	

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
drug (Sucrose)				
chemical compound,				
drug (LIVE/DEAD Fixable				
Aqua Dead Cell Stain Kit, for 405 nm excitation)	Aqua	ThermoFisher Scientific	Cat # L34957	
chemical compound, drug (Isoflurane, USP)	Isoflurane	Piramal	CAS 26675-46-7	
chemical compound, drug (4',6-Diamidino-2- Phenylindole, Dihydrochloride)	DAPI	ThermoFisher Scientific	CAS 28718-90-3; Cat # 1306	
chemical compound, drug (Fluoromount-G, with DAPI)	Fluoromount-G, with DAPI	ThermoFisher Scientific	Cat # 00-4959-52	
antibody (CD117 (c-Kit) Monoclonal Antibody (2B8), Biotin)	c-Kit-Biotin	eBioscience	RRID: AB_466569 ; Cat # 13-1171-82	
antibody (FceR1 alpha Monoclonal Antibody (AER-37 (CRA1)), PE, eBioscience)	FceRI-PE	eBioscience	RRID: AB_10804885 ; Cat # 12-5899-42	
antibody (CD49b (Integrin alpha 2) Monoclonal Antibody (DX5), PE-Cyanine7, eBioscience)	CD49b-PECy7	eBioscience	RRID: AB_469667 ; Cat # 25-5971-82	
antibody (Anti-Siglec-F-APC	SiglecF-APC	Miltenyi Biotech	RRID: AB_2653441 ; Cat # 130-112-333	

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
mouse (clone: REA798))				
antibody (Streptavidin FITC)	SA-FITC	eBioscience	RRID: AB_11431787 ; Cat # 11-4317-87	
antibody (Ly-6C Monoclonal Antibody (HK1.4), PerCP-Cyanine5.5, eBioscience)	Ly6C-PerCP	eBioscience	RRID: AB_1518762 ; Cat # 45-5932-82	
antibody (Pacific Blue anti-mouse/human CD11b Antibody)	CD11b-PB	BioLegend	RRID: AB_755985 ; Cat # 101223	
antibody (Brilliant Violet 785 anti-mouse Ly-6G Antibody)	Ly6G-BV785	BioLegend	RRID: AB_2566317 ; Cat # 127645	
antibody (CD45.2 Monoclonal Antibody (104), Alexa Fluor 700, eBioscience)	CD45.2-AF700	eBioscience	RRID: AB_657752 ; Cat # 56-0454-82	
software, algorithm (Igor Pro version 6.3)	IgorPro	WaveMetrics	https://www.wavemetrics.com/order/order_igordownloads6.htm	
software, algorithm (Microsoft Excel 2011)	Microsoft Excel	Microsoft	https://www.microsoft.com/en-us/store/d/excel-2016-for-mac/	
software, algorithm (pClamp 10)	pClamp	Axon	http://mdc.custhelp.com/app/answers/detail/a_id/18779/~/axon%E2%84%A2-	

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
software, algorithm (MetaFluor 7.8)	MetaFluor	Molecular Devices	pclamp%E2%84%A2-10-electrophysiology-data-acquisition-%26-analysis-software	
software, algorithm (MATLAB)	MATLAB	MathWorks	https://www.mathworks.com/downloads/	
software, algorithm (FIJI)	FIJI	NIH	https://imagej.net/Fiji/Downloads	
software, algorithm (LabChart Software)	LabChart Software	AD Instruments	https://www.adinstruments.com/products/labchart	
software, algorithm (Graphpad Prism 7)	Graphpad Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/	
software, algorithm (FlowJo 10.4.2)	FlowJo	FlowJo	https://www.flowjo.com/solutions/flowjo/downloads	
software, algorithm (custom)	custom-made software in MATLAB	this paper	NA	https://gi.SpikeSo(HoffmaarchivedelifeSciSpikeSo

Reagent type (species or resource)	Designation	Source or reference	Identifiers	Add
other (Bovine serum albumin, cold ethanol fraction, pH 5.2, \geq 96%)	BSA	Sigma-Aldrich	Cat # A4503	CAS 9048-46-8;
other (Isolectin B4 (Bandeireia simplicifolia), FITC- conjugate)	IB4-FITC; IB4	Enzo Life Sciences	MC05	Cat # ALX-650-001F-
other (Normal Goat Serum)	NGS	Abcam	Cat # ab7481	
peptide, recombinant protein (δ - theraphototoxin- Hm1a)	Hm1a	other; https:// www.ncbi.nlm.nih.gov/ pmc/articles/ PMC4919188/ PMID: 4919188	NA	obtained laborato of David

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Behavioral studies and mice

Slpr3^{mcherry/+} and *Slpr3^{-/-}* mice were obtained from Jackson Laboratory and backcrossed to C57bl6/J. Wherever possible, wild-type/heterozygous (*Slpr3*) littermate controls were used in behavioral experiments. Mice (20–25 g) were housed in 12 hr light-dark cycle at 21°C. Mice were singly housed one week prior to all behavioral experiments and were between 8–10 weeks at the time of the experiment. All mice were acclimated in behavioral chambers (IITC Life Sciences) on two subsequent days for 1 hr prior to all behavioral experiments.

Itch and acute pain behavioral measurements were performed as previously described ([Morita et al., 2015](#); [Wilson et al., 2013](#); [Tsunozaki et al., 2013](#)). Mice were shaved one week prior to itch behavior. Compounds injected: 500 μ M TY 52156 (Tocris), 50 μ M SKI II (Tocris), 0.2–10 μ M S1P (Tocris, Avanti Polar Lipids), 50 mM chloroquine (Sigma), and 27 mM histamine (Tocris) in PBS with either 0.01–0.1% Methanol- (S1P) or 0.1–0.5% DMSO-PBS (all other compounds) vehicle controls. Pruritogens were injected using the cheek model (20 μ L) of itch, as previously described ([Shimada and LaMotte, 2008](#)). Behavioral scoring was performed while blind to experimental condition and mouse

genotype. All scratching and wiping behavior videos were recorded for 1 hr. Itch behavior was scored for the first 30 min and acute pain was scored for the first five minutes. Bout number and length were recorded.

For radiant heat and von Frey hypersensitivity behavior, drugs were injected intradermally into the plantar surface of the hindpaw (20 µL). Radiant heat assays were performed using the IITC Life Science Hargreaves test system. Mechanical threshold was measured using calibrated von Frey monofilaments (Touch Test) on a metal grate platform (IITC). Von Frey was performed as previously described ([Tsunozaki et al., 2013](#); [Chaplan et al., 1994](#)) using the up-down method ([Dixon, 1965](#)) while blinded to compound injected and genotype, or a descending force-series of 4 trials per force from 0.4 g to 6 g. Valid responses for both von Frey and radiant heat included fast paw withdrawal, licking/biting/shaking of the affected paw, or flinching. For radiant heat and von Frey, mice were allowed to acclimate on platform for 1 hr before injection. Measurements were taken 15 min pre-injection and 20–30 min post-injection for all compounds used.

The pinprick assay ([Duan et al., 2014](#)) was conducted on a von Frey testing platform (IITC). The mouse hindpaw was poked with a 31 g syringe needle without breaking the skin to induce fast acute mechanical pain. Each paw was stimulated 10 times with the needle, with five minutes rest in between trials, and the % withdrawal (fast withdrawal, licking/biting/shaking of paw, squeaking, and/or flinching) was calculated from the total number of trials.

The tape assay was conducted according to previously described methods ([Ranade et al., 2014](#)). Number of attempts to remove a 3 cm piece of lab tape was recorded for 10 min after manual tape application to the rostral back. Scorer and experimenter were blinded to genotype.

For righting reflex measurements, age-matched *Slpr3^{-/-}* and ^{+/+} P6-7 neonates were used. Briefly, pups were overturned one at a time on the home cage lid while experimenter was blinded to genotype. The time to righting was measured to the nearest 1/10th of a second with a stopwatch.

For the CFA model of hypersensitivity, mice were lightly anesthetized with isoflurane (2%) and injected with 15 µL CFA (Sigma) into one hindpaw using a Hamilton syringe (30 g) at 5pm. Radiant heat latencies and von Frey 50% withdrawal thresholds were recorded one day prior to CFA, the morning of CFA (prior to injection), and one and two days post-CFA. Von Frey measurements were acquired before radiant heat latencies, and mice were allowed a one-hour recovery period in home cage with access to food and water in between testing. Both ipsilateral and contralateral paw were measured. Experimenter was blind to genotype for injections and recording.

All behavior experiments were carried out using age-matched or littermate cohorts of male mice and conducted between 8 am and 1 pm. Mice were tested in 4-part behavior chambers (IITC Life Sciences) with opaque dividers (TAP Plastics) with the exception of righting reflex measurements. Scratching and wiping behaviors were filmed from below using high-definition cameras. All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, Berkeley Animal Care and Use

Committee.

In situ hybridization (ISH)

Fresh DRG were dissected from 8 to 12 week old mice, flash frozen in OCT embedding medium, and sectioned at 14 µm onto slides. ISH was performed using Affymetrix Quantigene ViewISH Tissue 2-plex kit according to manufacturer's instructions with Type 1 (*S1pr3*) and Type 6 (all other) probes. The following probes against mouse mRNAs were created by Affymetrix and used for ISH: *S1pr3*, *Scn1a*, *Npy2r*, *Piezo2*, *Trpv1*, *Trpa1*.

Immunohistochemistry (IHC) of DRG

DRG were dissected from 8 to 12 week old adult mice and post-fixed in 4% PFA for one hour. DRG were cryo-protected overnight at 4°C in 30% sucrose-PBS, embedded in OCT, and then cryosectioned at 12 µm onto slides. Briefly, slides were washed 3x in PBST (0.3% Triton X-100), blocked in 2.5% horse serum +2.5% BSA PBST, and incubated overnight at 4°C in 1:1000 primary antibody in PBST +0.5% horse serum +0.5% BSA. Slides were washed 3X in PBS then incubated 1–2 hr at RT in 1:1000 secondary antibody. Slides were washed 3X in PBS and mounted in Fluoromount-G +DAPI with No. 1.5 coverglass. Primary antibodies used: Rabbit anti-DsRed (Clontech #632496), Rabbit anti-S1PR3 (Abcam #38324; #108370), Mouse anti-NF200 (Sigma #N5389), Chicken anti-Peripherin (Abcam #39374). Secondary antibodies used: Goat anti-Mouse Alexa 488 (Abcam #150117), Goat anti-Chicken Alexa 488 (ThermoFisher #A11039), Goat anti-Rabbit Alexa 594 (Invitrogen #[R37117](#)). Isolectin B4 (IB4)-FITC (Enzo Life Sciences #ALX-650-001F-MC05) was also used. Slides were mounted in Fluoromount with No. 1.5 coverglass. Imaging of DRG ISH and IHC experiments, and all live-cell imaging, was performed on an Olympus IX71 microscope with a Lambda LS-xl light source (Sutter Instruments). For DRG ISH and IHC analysis, images were analyzed using FIJI software. Briefly, DAPI-positive cells were circled and their fluorescence intensity (AFU) for all channels was plotted against cell size using Microsoft Excel software. Co-labeling analysis was performed using FIJI. Intensity thresholds were set based on the negative control (no probe) slide. Cells were defined as co-expressing if their maximum intensities exceeded the threshold for both channels of interest.

IHC of sectioned skin

Skin was dissected from 8 week old adult mice and post-fixed in 4% PFA for 30 min at RT. DRG were cryo-protected overnight at 4°C in 30% sucrose-PBS, embedded in OCT, and then sectioned at 18 µm onto slides. Briefly, slides were blocked in 5% normal goat serum in PBST (0.1% Triton X-100) and incubated overnight at 4°C in 1:1000 primary antibody in blocking buffer. Slides were washed 3X in PBS then incubated 45 min at RT in 1:1000 secondary antibody. Slides were washed 5X in PBS and mounted in Fluoromount-G +DAPI with No. 1.5 coverglass. Primary antibodies used: Rabbit anti-DsRed (Clontech #632496), Chicken anti-NefH (Abcam #4680), Chicken anti-β-tubulin III (Abcam

#107216), mouse anti-PGP9.5 (Abcam #8189). Secondary antibodies used: Goat anti-Mouse Alexa 488 (Abcam #150117), Goat anti-Rabbit Alexa 594 (Invitrogen #[R37117](#)), Goat anti-Chicken Alexa 488 (ThermoFisher #A11039). For co-localization analysis, only fibers for which >50% of the length of the visible fiber contained co-localized (white) pixels were counted. Image analysis was performed using FIJI.

Whole mount skin IHC

Staining was performed according to [Marshall et al. \(2016\)](#). Briefly, 8-week-old mice were euthanized and the back skin was shaved, depilated, and tape-stripped. The removed skin was fixed overnight in 4% PFA, then washed in PBS (3X for 10 min each). Dermal fat was scraped away with a scalpel and skin was washed in PBST (0.3% Triton X-100; 3X for two hours each) then incubated in 1:500 primary antibody (Rabbit anti DsRed: Clontech #632496; Chicken anti-Nefh: Abcam #4680) in blocking buffer (PBST with 5% goat serum and 20% DMSO) for 5.5 days at 4°C. Skin was washed as before and incubated in 1:500 secondary antibody (Goat anti-Rabbit Alexa 594; Invitrogen #[R37117](#); Goat anti-Chicken Alexa 488; ThermoFisher #A11039) in blocking buffer for 3 days at 4°C. Skin was washed in PBST, serially dried in methanol: PBS solutions, incubated overnight in 100% methanol, and finally cleared with a 1:2 solution of benzyl alcohol: benzyl benzoate (BABB; Sigma) before mounting between No. 1.5 coverglass. Sectioned and whole mount skin samples were imaged on a Zeiss LSM 880 confocal microscope with OPO using a 20x water objective. Image analysis was performed using FIJI.

Cell culture

Cell culture was carried out as previously described ([Wilson et al., 2011](#)). Briefly, neurons from dorsal root ganglia (2–8 week old adults) or trigeminal ganglia (P0) were dissected and incubated for 10 min in 1.4 mg ml⁻¹ Collagenase P (Roche) in Hanks calcium-free balanced salt solution, followed by incubation in 0.25% standard trypsin (vol/vol) STV versene-EDTA solution for 2 min with gentle agitation. Cells were then triturated, plated onto Poly D-Lysine coated glass coverslips and used within 20 hr. Media: MEM Eagle's with Earle's BSS medium, supplemented with 10% horse serum (vol/vol), MEM vitamins, penicillin/streptomycin and L-glutamine.

Calcium imaging

Ca²⁺ imaging experiments were carried out as previously described ([Wilson et al., 2011](#)). Cells were loaded for 60 min at room temperature with 10 μM Fura-2AM supplemented with 0.01% Pluronic F-127 (wt/vol, Life Technologies) in a physiological Ringer's solution containing (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 CaCl₂, 2 MgCl₂ and 10 D-(+)-glucose, pH 7.4. All chemicals were purchased from Sigma. Acquired images were displayed as the ratio of 340 nm/380 nm. Cells were identified as neurons by eliciting depolarization with high potassium Ringer's solution (75 mM) at the end of each experiment. Responding neurons were defined as those having a > 15% increase from baseline ratio.

Image analysis and statistics were performed using automated routines in Igor Pro (WaveMetrics). Fura-2 ratios were normalized to the baseline ratio $F340/F380 = (\text{Ratio})/(\text{Ratio } t = 0)$.

In vitro electrophysiology

Electrophysiological experiments were carried out as previously described ([Wilson et al., 2011](#)). Briefly, recordings were collected at 5 kHz and filtered at 2 kHz (Axopatch 200B, pClamp software). Electrode resistance ranged between 1.5–5 M Ω . Internal solution contained 140 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 5 mM HEPES, 1 mM Na₂ATP, 100 μ M GTP, and 100 μ M cAMP (pH 7.4). Bath solution was physiological Ringer's solution. The pipette potential was canceled before seal formation. Cell capacitance was canceled before whole cell voltage-clamp recordings. For mechanonociceptors experiments, only cells which were visually identified as mCherry expressing and which had a capacitance between 40–80 pF were used. Rheobase was calculated as the smallest current step required to elicit an action potential using current steps of 50 pA. M currents were measured and analyzed using standard protocols for DRG neurons reported in the literature ([Schütze et al., 2016](#); [Crozier et al., 2007](#); [Zheng et al., 2013](#)). Experiments were carried out only on cells with a series resistance of less than 30 M Ω . Analysis of electrophysiology data was performed in pClamp and IgorPro.

Ex vivo skin-nerve electrophysiology

Touch-evoked responses in the skin were recorded after dissecting the hind limb skin and saphenous nerve from 7 to 10 week old mice, according to published methods ([Wellnitz et al., 2010](#); [Maksimovic et al., 2014](#)). The skin was placed epidermis-side-up in a custom chamber and perfused with carbogen-buffered synthetic interstitial fluid (SIF) kept at 32°C with a temperature controller (model TC-344B, Warner Instruments). The nerve was kept in mineral oil in a recording chamber, teased apart, and placed onto a gold recording electrode connected with a reference electrode to a differential amplifier (model 1800, A-M Systems). The extracellular signal was digitized using a PowerLab 8/35 board (AD Instruments) and recorded using LabChart software (AD Instruments).

For these studies, we focused on A-mechanonociceptors (AMs). To identify responses from these afferents in mutant and control genotypes, we used a mechanical search paradigm with a fine glass probe. Afferents were classified as AMs according to the following criteria: (1) conduction velocity (approximately, one to (\leq 12 m/ s⁻¹), (2) medium-sized receptive fields, (3) sustained response to mechanical indentation ([Wellnitz et al., 2010](#); [Koltzenburg, 1997](#); [Zimmermann et al., 2009](#)).

Touch-sensitive afferents that did not meet these criteria were not analyzed further. Responses were classified as Adapting AMs if the ratio of mean firing rate in the dynamic phase of stimulation (first 0.2 s) to the static phase of stimulation (last 4.8 s) was greater than 2, and Non-Adapting AMs if the ratio was less than or equal to 2. Non-

responders (**Figure 6F**) responded to suprathreshold mechanical stimulation with von Frey monofilaments (tip diameter <0.5 mm), but not to maximal controlled mechanical stimulation (256 mN, tip diameter 2 mm). All recordings and analyses were performed blind to genotype.

Mechanical responses were elicited with von Frey monofilaments and a force controlled custom-built mechanical stimulator. Mechanical thresholds were defined as the lowest von Frey monofilament to reliable elicit at least one action potential. Force controlled mechanical stimuli were delivered using a computer controlled, closed-loop, mechanical stimulator (Model 300C-I, Aurora Scientific, 2 mm tip diameter). Low-pass filtered, 5 s long, length control steps (square wave) simultaneously delivered with permissive force control steps (square wave) were generated using LabChart software (AD Instruments). An arbitrarily selected force step-and-hold protocol (8, 32, 4, 64, 128, 16, 256 mN) was delivered to all fibers. The period between successive displacements was 60 s.

Conduction velocity was measured by electrically stimulating identified receptive fields. Spike sorting by principal component analysis (PCA) and density based clustering, and data analysis was performed off-line with custom-made software in MATLAB. Statistics were performed in Prism.

Flow cytometry of CFA-treated hind paws

CFA injections were performed as described above. Briefly, hindpaw skin and underlying fascia of treated and PBS-injected paws were removed from freshly euthanized mice. Skin was placed in RPMI media (Gibco) on ice before mincing with dissection scissors. Digestions were performed for 90 min at 37°C on a rotating platform in 1 mL RPMI supplemented with 1:1000 DNaseI enzyme (Roche) and one unit LiberaseTM (Roche). Skin was then filtered through 70 µm nylon mesh (Falcon), washed in RPMI, and resuspended in PBS for Aqua Live-Dead staining. Samples were then transferred to FACS buffer (PBS with 0.5% FBS and 2 mM EDTA), blocked, then surface stained with the following antibodies: cKit-Biotin, FceRI-PE, CD49b-PECy7, SiglecF-APC, SA-FITC, Ly6C-PerCP, CD11b-PB, Ly6G-BV785, CD45.2-AF700. Compensation tubes (single-stain) were prepared for each fluorophore using positive and negative control beads. A spleen from a wild-type animal was also prepared by crushing between frosted glass slides, straining through 70 µm nylon mesh, and lysing of erythrocytes in ACK (Gibco). A portion of spleen sample was heat-killed for 10 min at 65°C and stained with Aqua viability stain and set aside. The rest of the spleen was stained normally with the other skin samples. Samples were then run through a flow cytometer (BD Fortessa). Data were analyzed using FlowJo (Prism) and Microsoft Excel. Neutrophils were defined as live single cells with the following staining profile: CD45.2⁺/CD11b⁺/Ly6G⁺/Ly6C⁺. Inflammatory monocytes were defined as the following: CD45.2⁺/CD11b⁺/Ly6G⁻/Ly6C^{high}. Total number of immune cells was reported, rather than percentage of total, since neither genotype differed significantly in total number of live cells or total number of CD45.2⁺ immune cells.

Statistical analyses

All statistical analyses, except for skin nerve data (see above), were performed using IgorPro software or Microsoft Excel. Values are reported as the mean \pm SEM where multiple independent experiments are pooled and reported (for whole cell electrophysiology), and mean \pm SD where one experiment was performed with multiple wells (for calcium imaging) or mice (for behavior). For comparison between two groups, Student's unpaired 2-tailed t-test was used. A paired t-test was employed only for measurements within the same biological replicate and after a given treatment. For single-point comparison between >2 groups, a one-way ANOVA followed by appropriate post hoc test was used, depending on comparison. For the time course comparison between two groups, 2-way ANOVA was used and single comparison p-values were derived using Tukey's HSD or appropriate statistical test, depending on comparison. Number of mice or samples required to attain significance was not calculated beforehand, and where multiple statistical tests were performed, a Bonferroni correction was applied. In figure legends, significance was labeled as: n.s., not significant, $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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Additional information

Competing interests

No competing interests declared.

Author contributions

Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing, Performed behavior, immunostaining, whole cell electrophysiology, calcium imaging, and ISH.

Data curation, Formal analysis, Investigation, Visualization, Methodology, Writing—review and editing, Performed ex vivo recordings.

Data curation, Investigation, Writing—review and editing, Performed ISH experiments.

Investigation, Writing—review and editing, Performed ex vivo recordings.

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Ethics

Animal experimentation: All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, Berkeley Animal Care and Use Committee (Protocol Number: AUP-2017-02-9550).

Additional files

Supplementary file 1. Co-ISH quantification for sectioned DRG from adult wild-type mice.

Related to [Figure 3](#).

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Decision letter

Editor: David D Ginty¹

In the interests of transparency, eLife includes the editorial decision letter and accompanying author responses. A lightly edited version of the letter sent to the authors after peer review is shown, indicating the most substantive concerns; minor comments are not usually included.

[Editors' note: this article was originally rejected after discussions between the reviewers, but the authors were invited to resubmit after an appeal against the decision.]

Thank you for submitting your work entitled "The signaling lipid sphingosine 1-phosphate regulates mechanical pain" for consideration by *eLife*. Your article has been reviewed by three peer reviewers, and the evaluation has been overseen by a Reviewing Editor and Gary Westbrook as the Senior Editor. The following individuals involved in review of your submission have agreed to reveal their identity: Bruce P Bean (Reviewer #3).

Our decision has been reached after consultation between the reviewers. Based on these discussions and the individual reviews below, we regret to inform you that your work will not be considered further for publication in *eLife*. Although the reviewers found merit in the study, a number of serious concerns were raised, and it is our editorial opinion that these concerns cannot be adequately addressed in the time frame set by *eLife* policy.

Reviewer #1:

In this manuscript, the authors characterized the role of sphingosine 1-phosphate receptor 3 (S1PR3), a candidate gene from an earlier study to screen for genes enriched in mechanosensory neurons, specifically in mechanical pain sensation. They show that (1) S1PR3 knockout mice show decreased sensitivity for noxious but not innocuous mechanical pain stimuli, (2) injection of either an S1PR3 antagonist or a kinase inhibitor that blocks S1P production also increases withdrawal thresholds, (3) S1PR3 expression in DRG partially overlaps with Npy2r, Piezo2, and Nav1.1, which marks mechanosensory DRG neurons, (4) Adelta mechanosensitive fibers show decreased firing rates upon mechanical stimulation in ex vivo skin nerve preparations, and (5) S1P application blocks KCNQ2/3-mediated tail currents in dissociated DRG neuron recordings. While these studies identify an interesting new pathway that sets mechanical pain thresholds, and the authors performed a number of convincing experiments to suggest a role for S1P signaling in Adela fiber physiology, there are a few unresolved questions about the data presented.

1) Mechanical sensation usually occurs very quickly, while GPCR signaling is usually much slower. For behavior

assays, the authors injected pharmacological reagents at least 30 minutes ahead of time. Thus, it doesn't seem to be very possible that S1P/S1PR3 signaling occurs when VFH was applied to the skin and modulate the mechanical sensitivity in ms. It is more likely that there is a constant baseline ongoing S1P/S1PR3 signaling that helps to set the mechanical pain threshold. How does it exactly happen? Where is the resource of this constant ligand? The authors will need to clarify their ideas/model more in the text.

2) Much of the evidence for the author's model relies on a global knockout mouse. As discussed above, if S1P/S1PR3 signaling is ongoing, would a global null mouse cause developmental deficit or other nonspecific effects that may explain the phenotype? It is also unclear the cell types/location responsible for the phenotype. While the authors do show an acute behavioral effect of a local injection of a S1PR3 antagonist and a blocker of S1P production, it does not exclude that some phenotypes they observed come from developmental deficits. Their overall model would be strengthened by a more careful characterization of the mutant mice. Use of a conditional knockout could address these concerns, either by an inducible Cre to delete S1PR3 in adulthood or restricting deletion to AM fibers. If this is not feasible, the authors could better characterize the knockout mouse, such as expression of known pain genes in DRG neurons or AM fibers, peripheral and central innervations, etc., and the developmental expression of S1PR3 in DRG neurons. For example, if S1PR3 is only expressed in adult DRG neurons, this developmental concern will be eased.

3) The characterization of S1PR3 expressing cells was only conducted to show its expression in AM neurons. It is fairly clear that this gene is expressed in other populations of DRG neurons. Based on the characterization provided, less than half of S1pr3+ neurons are AM fibers, while ~25% are Trpv1+ peptidergic neurons (although this data is not shown in the figure). Do LTMR types express S1pr3? A more thorough characterization of S1pr3 expression (NFH, peripherin, IB4, and other markers) and quantification would be helpful. Why don't the KO mice display deficits in other populations of expressing neurons? Some discussion/thoughts in this direction would be nice.

Reviewer #2:

The current study has demonstrated that the bioactive lipid sphingosine 1-phosphate (S1P) and S1P Receptor 3 (S1PR3) are critical regulators of acute mechanical pain. Although the finding is largely consistent with a previous study (Camprubi-Robles et al., 2013), the authors have provided more detailed mechanisms underlying S1P on DRG neurons. They found that S1P affected A-delta mechanonociceptor excitability by modulating of KCNQ2/3 channels.

Although the study is potentially interesting, there are some concerns need to be addressed.

The result of S1P injection not affecting mechanosensitivity (Figure 2A) is very surprising. The authors explained that the endogenous S1P is sufficient to maximally exert its effect on S1PR3-dependent mechanical pain. However, a previous study has shown that S1P injection induced a dose dependent nociceptive response (Camprubi-Robles et al., 2013). The author should do a more careful dose response study. Another experiment can be done is that co-injection of

SKI II (sphingosine kinase inhibitor) with S1P to determine whether S1P increase the attenuated mechanosensitivity by SKI II. If it is true, it suggests that S1P constitutively activates S1PR3.

The same previous study (Camprubi-Robles et al., 2013) has shown that S1PR3 *in situ* is expressed in nearly all DRG neurons and the staining is completely gone in S1PR3 knockout DRG (Figure 6A in that study). The authors should employ a different approach to reconcile the discrepancy such as using S1PR3mcherry/+ mice.

Why did they use P0 TG of S1PR3mcherry/+ mice to do Ca²⁺ imaging in response to Hm1a (Figure 3F)? Why not use adult DRG neurons? mCherry imaging in Figure 3F is not convincing. A better image from DRG should be provided.

Only S1PR3 heterozygous mice were used in AM recording experiments. Wild type mice should be used to see the full extent of S1PR function (Figure 4).

Reviewer #3:

This manuscript reports on the involvement of the bioactive lipid sphingosine-1-phosphate (S1P) in regulating DRG neuron excitability and pain behavior acting via the S1P receptor 3 (S1PR3). The key findings are that S1PR3-/- mice have diminished sensitivity to mechanical pain (von Frey hairs), that SP1R3 mRNA is expressed in ~40-50% of all DRG neurons, and that in SP1R3-/- mice, the response to von Frey hairs is diminished in single unit recordings from skin-nerve preparations. In addition, the authors find that S1P applied to cultured DRG neurons enhances excitability, which they propose results from closure of Kv7 channels. They propose that *in vivo*, endogenous S1P is high enough to produce basal closure of Kv7 channels.

The manuscript has interesting results. However, it has two serious deficiencies. One is its failure to acknowledge how much the findings overlap with previously published work, most notably with Camprubi-Robles et al., 2013. This paper is cited in the manuscript but only in a negative manner, saying that the antibodies used in that paper were found in the present work to be non-specific. There is failure to acknowledge the many results in the Camprubi-Robles paper that overlap with the results in this manuscript, including the reduced sensitivity to mechanical pain in SP1R3-/- mice, and demonstration using ISH of mRNA in many DRGS neurons (which included a nice use of SP1R3-/- mice as a control, just as in the current manuscript). Also, the authors fail to mention a series of papers from the Nicol lab showing involvement of S1P and S1PRs in pain, including showing that S1P enhances excitability of DRG neurons by reducing a potassium current and also enhancing TTX-resistant sodium current (Zhang et al., 2006) and that this effects is diminished using siRNA targeted to S1PR1 and SIPR2 receptors (Li et al., 2015).

A second major deficiency is in the analysis of the ionic mechanism underlying the increase in excitability reported here (and in the previous papers) by S1P applied to DRG neurons. The authors attribute this to closure of Kv7 channels (M-

current). The evidence for this particular mechanism is very weak. The standard protocol for M-current is recording the slowly-deactivating outward tail currents when stepping from a steady holding potential of around -40 mV or -30 mV to around -60 mV. Here, the authors measure tail currents at -80 mV following steps to +100 mV. It is very hard to interpret these currents. It does not make sense to try to measure KV7 currents repolarizing to -80 mV, because the driving force on K is so small. In any case, I could not understand in Figure 5D how the authors were making the measurements or what the currents are. There is a sequence of fast-decaying current followed by a slowly-increasing outward current. This looks nothing like a recording of M-current (which would be a monotonically slowly-decaying outward current). The only evidence for it being mediated by Kv7 channels is occlusion by 100 μ M linopirdine. This is of doubtful selectivity. The standard inhibitor of M-current (Kv2/Kv3) is XE-991, which acts with an IC50 of less than 1 μ M.

This attribution of the increase in excitability by S1P to Kv7 inhibition is all the more unconvincing in light of the previous work done, which is not mentioned. Zhang et al. (2006, above) found that S1P both inhibited a high-threshold voltage-activated K current (clearly not Kv7 from its voltage dependence) and also enhanced TTX-resistant sodium channels, and Camprubi-Robles found that S1P induces a large inward current at holding potential of -80 mV (clearly not closing Kv7 channels, which would already be closed at this voltage), which they attributed to activation of chloride channels. The overall impression from all the work is that multiple conductances are affected. The evidence for closure of Kv7 channels being important is probably the least strong of any of the conductances implicated.

In my opinion, the manuscript cannot be published in its present form, based on the lack of discussion (and often, even acknowledgement) of closely related previous work and the weakness of the attribution of the S1P effects exclusively to inhibition of Kv7 channels.

[Editors' note: what now follows is the decision letter after the authors submitted for further consideration.]

Thank you for resubmitting your work entitled "The signaling lipid sphingosine 1-phosphate regulates mechanical pain" for further consideration at *eLife*. Your revised article has been favorably evaluated by Gary Westbrook (Senior editor), a Reviewing editor, and three reviewers.

The manuscript has been improved but there are some remaining issues that need to be addressed before acceptance, as outlined below. These are relatively minor and upon resubmission the changes to the manuscript will be evaluated by the Reviewing Editor.

1) The new experiments with a conventional voltage protocol for M-current are convincing, and the new panels in Figure 5 are very nice. However, now it is hard for the reader to understand Figure 5C and 5D carried over from the previous version, or what the difference in protocol was for the "tail currents" plotted in Figure 5C and 5D versus those in Figure 5E. The voltage protocols for Figure 5C and Figure 5D are not shown, and it is not explained where tail

current was measured. This should be clarified in the Figure legend (based on the previous version, presumably currents were activated by steps to +80 or +100, but I was unclear then and am still unclear at what time after the repolarization to -80 mV the tail currents were measured.). The figure might now work better leaving out Figure 5C and 5D (or moving them to the Supplementary figure) but at the least the protocols and measurements need to be explained clearly.

2) It was hard to understand Figure 6E until going deep into Materials and methods section. It is not at all obvious from the traces in the hets why the top is classified as "non-adapting" and the bottom as "adapting". Both keep firing throughout the stimulus and both show much slower firing in the second half of the stimulus. In fact, the firing during the later stimulus is faster in the "non-adapting" fiber than in the "adapting" fiber, which seems confusing. One has to go to the Materials and methods section to realize that the distinction is when frequency falls by 2-fold from the initial frequency to the end, so the initial frequency must be much faster in the bottom trace than the top. It would be much clearer if the plots included a trace of frequency averaged over 200 ms intervals or something similar. If that doesn't fit well into the figure, at least the main text should explain the criterion that is being used, and maybe say something like "the top trace shows an example of a non-adapting fiber, in which the initial frequency of xx Hz fell less than 2-fold over the 5-sec stimulus, to xx Hz. The bottom trace shows an example of a non-adapting fiber, in which the initial frequency of xx Hz fell to xx HZ by the end of the stimulus."

3) Figure 5—figure supplement 1E. "(Left)% Δ in peak instantaneous sodium current after S1P" – one assumes that it isn't "instantaneous" current but just "peak sodium current" during the depolarization that is plotted.

4) Subsection "Endogenous S1P mediates acute mechanical pain" "that increased of S1P does not evoke..." – delete "of".

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Author response

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[Editors' note: the author responses to the first round of peer review follow.]

Reviewer #1:

In this manuscript, the authors characterized the role of sphingosine 1-phosphate receptor 3 (S1PR3), a candidate gene from an earlier study to screen for genes enriched in mechanosensory neurons, specifically

in mechanical pain sensation. They show that (1) S1PR3 knockout mice show decreased sensitivity for noxious but not innocuous mechanical pain stimuli, (2) injection of either an S1PR3 antagonist or a kinase inhibitor that blocks S1P production also increases withdrawal thresholds, (3) S1PR3 expression in DRG partially overlaps with Npy2r; Piezo2, and Nav1.1, which marks mechanoinsensitive DRG neurons, (4) Adelta mechanosensitive fibers show decreased firing rates upon mechanical stimulation in ex vivo skin nerve preparations, and (5) S1P application blocks KCNQ2/3-mediated tail currents in dissociated DRG neuron recordings. While these studies identify an interesting new pathway that sets mechanical pain thresholds, and the authors performed a number of convincing experiments to suggest a role for S1P signaling in Adela fiber physiology, there are a few unresolved questions about the data presented.

1) Mechanical sensation usually occurs very quickly, while GPCR signaling is usually much slower. For behavior assays, the authors injected pharmacological reagents at least 30 minutes ahead of time. Thus, it doesn't seem to be very possible that S1P/S1PR3 signaling occurs when VFH was applied to the skin and modulates the mechanical sensitivity in ms. It is more likely that there is a constant baseline ongoing S1P/S1PR3 signaling that helps to set the mechanical pain threshold.

We agree with reviewer 1 that it is unlikely that mechanical force triggers rapid S1P release, and instead, agree that our data support a model wherein S1P/S1PR3 signaling constitutively sets the mechanical threshold. Consistent with this model, the dose-dependent effects of S1P that we measured on mechanonociceptors in vitro (Figure 5—figure supplement 1F) are saturated within the reported baseline levels of S1P in tissues in vivo (~100 nM; Ramos-Perez et al. 2015; Pappu et al. 2007). In addition, we provide new data in support of this model showing that 75-200 nM S1P is sufficient to reverse the mechanical hypersensitivity triggered by endogenous S1P depletion in vivo (Figure 2E; see response to Reviewer 2, comment 2). We have amended the Results and Discussion to highlight the data in support of a constitutive model.

How does it exactly happen? Where is the resource of this constant ligand? The authors will need to clarify their ideas/model more in the text.

We agree this is an interesting question. Indeed, this is a major research topic in immunology, where the role of S1P is well established. While we can't answer this question, we have added the following discussion regarding the cellular source of S1P:

"What is the local source of S1P in the skin that constitutively modulates mechanical pain? Even in the mature field of S1P signaling in the vascular and immune systems, the cellular source of S1P, while an intriguing question, remains unclear. All cells in the body, including somatosensory neurons, immune cells, and skin cells, express sphingosine kinases 1 and 2, which are essential for S1P production (42). Deletion of both kinases is lethal and attempts to conditionally knockout these kinases fail to completely eliminate S1P in tissues (43). While RNA seq data suggests that

somatosensory neurons contain all of the enzymatic machinery required to produce and export local S1P (19, 44) future work will be needed to identify the key cell types that are important for maintaining baseline S1P levels in the skin and regulating mechanical sensitivity.”

2) Much of the evidence for the author's model relies on a global knockout mouse. As discussed above, if S1P/S1PR3 signaling is ongoing, would a global null mouse cause developmental deficit or other nonspecific effects that may explain the phenotype? It is also unclear the cell types/location responsible for the phenotype. While the authors do show an acute behavioral effect of a local injection of a S1PR3 antagonist and a blocker of S1P production, it does not exclude that some phenotypes they observed come from developmental deficits. Their overall model would be strengthened by a more careful characterization of the mutant mice. Use of a conditional knockout could address these concerns, either by an inducible Cre to delete S1PR3 in adulthood or restricting deletion to AM fibers.

We agree that conditional knockouts are best, but have made several attempts to generate these animals using a commercially-available S1PR3 “knockout-first” strain that have thus far failed. While we will pursue the creation of a new floxed line, we believe that our discovery of S1PR3 as a key mediator of mechanical pain in the global knockout is of immediate significance, given that we know little about the molecular mechanisms underlying mechanical pain, but have provided additional, new data on the global knockout to support our claims (see below).

If this is not feasible, the authors could better characterize the knockout mouse, such as expression of known pain genes in DRG neurons or AM fibers, peripheral and central innervations, etc., and the developmental expression of S1PR3 in DRG neurons. For example, if S1PR3 is only expressed in adult DRG neurons, this developmental concern will be eased.

We agree that a more extensive characterization of the global KO would better support our findings. Indeed, we have seven new experiments, which suggest normal somatosensory neuron development in knockout animals: 1) the average diameters of S1PR3 KO and wild-type DRG cells are not statistically different (Figure 3—figure supplement 1D), and neither are the diameters of *Trpv1*⁺C nociceptors (Figure 3—figure supplement 1D) or NF200⁺ myelinated neurons (Figure 3—figure supplement 1G). 2) S1PR3 KO mice are not significantly different from WT animals with respect to IB4, NF200, or Peripherin staining of sectioned DRG (Figure 3—figure supplement 1G). 3) Expression of *Trpv1* and *Trpa1* transcripts is similar in S1PR3 KO and wild-type DRG sections (Figure 3—figure supplement 1B). 4) Similar proportions of wild type and S1PR3 KO sensory neurons respond to capsaicin in ratiometric calcium imaging (Figure 3—figure supplement 1H). 5) PGP9.5, B-tubulin III, and NefH staining in S1PR3 KO skin displays expected patterns of innervation (see Results for statistical comparison). 6) S1PR3 KO and wild-type animals display similar proportions of A-beta, A-delta (including AM nociceptors), and C fibers as measured by conduction velocity in ex vivo skin-nerve recordings (Figure 6—figure supplement 1B). Thus while we see a reduction in mechanically sensitive AM fibers by controlled force stimulation (Figure 6E-F), we observe them in normal proportions based on conduction velocity and

suprathreshold von Frey stimulation. 7) We show that the mCherry reporter animals display no S1PR3 expression in epidermal and dermal cells (Figure 3D-F), and single-cell RNA seq of a diverse array of mouse skin cells corroborates this lack of expression (Joost et al. 2016). In addition, we also performed a battery of behavioral tests and found no baseline defects in sensations mediated by proprioceptors, low-threshold mechanoreceptors, thermal nociceptors, or pruriceptors in the KO animals (Figure 1).

These findings in the global knockout animal suggest that baseline S1P/S1PR3 signaling is selectively required for mechanical pain and does not affect other modalities. Overall, these experiments suggest that the phenotypes we observe in S1PR3 KO are not due to generalized defects in somatosensory development or function. Beyond the somatosensory system, S1PR3 KO animals display normal immune cell distribution and recruitment in a model of inflammatory pain (Figure 7—figure supplement 1A-B).

3) The characterization of S1PR3 expressing cells was only conducted to show its expression in AM neurons. It is fairly clear that this gene is expressed in other populations of DRG neurons. Based on the characterization provided, less than half of S1pr3⁺ neurons are AM fibers, while ~25% are Trpv1⁺ peptidergic neurons (although this data is not shown in the figure). Do LTMR types express S1pr3? A more thorough characterization of S1pr3 expression (NFH, peripherin, IB4, and other markers) and quantification would be helpful.

We have now performed a thorough ISH characterization to better characterize *S1pr3*-expressing cell types, including the use of new markers. These data show there are two main populations of *S1pr3*⁺ neurons: *Scn1a*⁺ mechanonociceptors (39.9% of *S1pr3*⁺), and *Trpv1*⁺ and/or *Trpa1*⁺ (67.1%) thermal nociceptors (Figure 3A-B). Staining of sectioned DRG, which is now included, shows that S1PR3 overlaps with Peripherin-expressing small-diameter neurons and IB4⁺ nociceptors as well as NF200⁺ medium-large myelinated neurons (Figure 3C,F).

Our whole mount and sectioned skin staining suggests that S1PR3 is not expressed in Merkel afferents or NefH⁺ hair follicle nerve endings (Figure 3D-E), which comprise subsets of LTMRs, but is rather expressed in NefH⁺ and NefH⁻ free nerve endings, which comprise AMs and C nociceptors, respectively.

Why don't the KO mice display deficits in other populations of expressing neurons? Some discussion/thoughts in this direction would be nice.

Our original manuscript dealt only with AMs and mechanical pain; however, we have also carried out an extensive set of experiments investigating mechanisms of S1P signaling in thermal pain. Consistent with previous studies (Camprubi-Robles et al. 2013) and our data showing expression of S1PR3 in thermal nociceptors, we see that elevated S1P triggers spontaneous pain that is absent in S1PR3 KO animals (Figure 2C-D), and S1P triggers excitation of capsaicin-sensitive nociceptors (Figure 4B-E). Importantly, we also go beyond these previous studies to show that elevated S1P and S1PR3

activity promote heat hypersensitivity in the CFA model (Figure 6A, C).

Reviewer #2:

The current study has demonstrated that the bioactive lipid sphingosine 1-phosphate (S1P) and S1P Receptor 3 (S1PR3) are critical regulators of acute mechanical pain. Although the finding is largely consistent with a previous study (Camprubi-Robles et al., 2013), the authors have provided more detailed mechanisms underlying S1P on DRG neurons. They found that S1P affected A-delta mechanonociceptor excitability by modulating of KCNQ2/3 channels.

Although the study is potentially interesting, there are some concerns need to be addressed.

The result of S1P injection not affecting mechanosensitivity (Figure 2A) is very surprising. The authors explained that the endogenous S1P is sufficient to maximally exert its effect on S1PR3-dependent mechanical pain. However, a previous study has shown that S1P injection induced a dose dependent nociceptive response (Camprubi-Robles et al., 2013). The author should do a more careful dose response study.

Our previous manuscript focused solely on the effects of S1P signaling on mechanical pain, which is saturated by baseline levels of S1P (~200 nM). However, consistent with Camprubi-Robles et al., that reported spontaneous pain to 500 μM S1P, our dose response study shows that elevated S1P (2 μM or higher) triggers spontaneous pain in the cheek model (Figure 2C). While this other study did not measure mechanical or thermal hypersensitivity after S1P injection, our new data now show that the spontaneous pain behaviors induced by elevated S1P are due to heat and not mechanical hypersensitivity and that hypersensitivity is dependent on S1PR3 (Figure 2A-B, D). Overall, our data reveal two key, novel findings. First, S1P/S1PR3 sets baseline mechanical pain thresholds. Second, elevated S1P levels, such as those produced during inflammation or disease, promote heat hypersensitivity, but not mechanical hypersensitivity.

Another experiment can be done is that co-injection of SKI II (sphingosine kinase inhibitor) with S1P to determine whether S1P increase the attenuated mechanosensitivity by SKI II. If it is true, it suggests that S1P constitutively activates S1PR3.

We thank the reviewer for this suggestion. We have performed this experiment *in vivo* and found that injection of S1P does indeed reverse SKI II-induced mechanical hyposensitivity and observe a maximal effect with 200 nM S1P. This finding that exogenous S1P is sufficient to reverse the mechanical hyposensitivity triggered by the depletion of endogenous S1P supports a model whereby S1P constitutively regulates mechanical pain.

The same previous study (Camprubi-Robles et al., 2013) has shown that S1PR3 in situ is expressed in nearly all DRG neurons and the staining is completely gone in S1PR3 knockout DRG (Figure 6A in that study). The authors should employ a different approach to reconcile the discrepancy such as using S1PR3mcherry/+ mice.

We have now performed a thorough ISH characterization to better characterize *S1pr3*-expressing cell types, including the use of new markers. These data show there are two main populations of *S1pr3*⁺ neurons: *Scn1a*⁺ mechanonociceptors (39.9% of *S1pr3*⁺), and *Trpv1*⁺ and/or *Trpa1*⁺ (67.1%) thermal nociceptors (Figure 3A-B). Staining of sectioned DRG, which is now included, shows that S1PR3 overlaps with Peripherin-expressing small-diameter neurons and IB4⁺ nociceptors as well as NF200⁺ medium-large myelinated neurons (Figure 3C,F). Our staining data complement findings from a recent single-cell RNA-seq study showing expression of *S1pr3* in two populations comprising a subset of DRG neurons: the NF200⁺ myelinated mechanoreceptors and peptidergic nociceptors (Usoskin et al. 2015).

Unfortunately, we cannot make direct comparisons of our data to theirs, as they did not examine co-localization with other markers. And although they conclude that *S1pr3* is expressed by “virtually all” somatosensory neurons, they did not quantify *S1pr3* expression in wild type ganglia. Regardless, we now provide extensive new data, which, combined with our previous data, supports selective expression of S1PR3 primarily in two neuronal subsets: mechanonociceptors and thermal nociceptors.

Why did they use P0 TG of S1PR3mcherry/+ mice to do Ca²⁺ imaging in response to Hm1a (Figure 3F)?

Why not use adult DRG neurons? mCherry imaging in Figure 3F is not convincing. A better image from DRG should be provided.

Our use of P0 TG was based on the original characterization of Hm1a by Osteen and colleagues (Osteen et al. 2016) who reported that PGE2 sensitization is required to see robust and sustained Hm1a-evoked calcium influx in adult DRG neurons, but that Hm1a robustly activates naive P0 trigeminal ganglion neurons (due to faster inactivation kinetics in the adult). Thus, after consultation with Dr. Julius, we used P0 neurons to avoid the added complication of pre-sensitizing neurons that could change excitability. We have added the following passages to the results (p.8) and figure caption (Figure 4), and also provide a clearer, more representative image to replace the previous version (Figure 4F):

“To this end, we asked whether the spider toxin Hm1a, a selective activator of AM nociceptors (28), triggers calcium influx in S1PR3-expressing trigeminal neurons. Indeed, we found that 44.2 ± 15.1% of Hm1a responsive neurons expressed mCherry (Figure 4F), consistent with our staining showing expression of *S1pr3* in AM nociceptors and the role of Hm1a-responsive neurons in mediating mechanical pain in vivo (28).”

“Figure 4F. (Left) Fura-2 AM calcium imaging after addition of 500 nM Hm1a in *S1pr3*^{mCherry/+} P0 TG neurons. TG

neurons were used in calcium imaging instead of adult DRG neurons because they maximally respond to Hm1a without prior PGE2 sensitization, due to slower sodium channel inactivation kinetics. Right-hand image indicates mCherry fluorescence. (Right)% of Hm1a-responsive P0 TG neurons that are mCherry+ (N = 1 animal, 1230 total neurons).”

Only S1PR3 heterozygous mice were used in AM recording experiments. Wild type mice should be used to see the full extent of S1PR function (Figure 4).

We now include recordings from S1PR3 WT AM fibers, which do not significantly differ from S1PR3 HET fibers in mechanically-evoked responses to controlled force stimulation (Figure 6-figure supplement 1A), and cite a number of other studies on AM fibers that report similar force-response curves for their wild-type recordings (Osteen et al. 2016, Garrison et al. 2015, Smith et al. 2013, Hillery et al. 2011, Kwan et al. 2009, McIlwrath et al. 2007). This now allows for the direct comparison of the effects of S1PR3 signaling across genotypes.

Reviewer #3:

This manuscript reports on the involvement of the bioactive lipid sphingosine-1-phosphate (S1P) in regulating DRG neuron excitability and pain behavior acting via the S1P receptor 3 (S1PR3). The key findings are that S1PR3-/- mice have diminished sensitivity to mechanical pain (von Frey hairs), that S1PR3 mRNA is expressed in ~40-50% of all DRG neurons, and that in S1PR3-/- mice, the response to von Frey hairs is diminished in single unit recordings from skin-nerve preparations. In addition, the authors find that S1P applied to cultured DRG neurons enhances excitability, which they propose results from closure of Kv7 channels. They propose that in vivo, endogenous S1P is high enough to produce basal closure of Kv7 channels.

1) The manuscript has interesting results. However, it has two serious deficiencies. One is its failure to acknowledge how much the findings overlap with previously published work, most notably with Camprubi-Robles et al., 2013. This paper is cited in the manuscript but only in a negative manner; saying that the antibodies used in that paper were found in the present work to be non-specific. There is failure to acknowledge the many results in the Camprubi-Robles paper that overlap with the results in this manuscript, including the reduced sensitivity to mechanical pain in S1PR3-/- mice...

We agree that it is essential to put our data in the context of this study, especially since the scope of our studies is quite different: a distinction that was not highlighted adequately in our original manuscript that only discussed AMs and mechanical pain. Our previous manuscript focused solely on the effects of S1P signaling on mechanical pain, which is saturated by baseline levels of S1P (~200 nM). However, consistent with Camprubi-Robles et al., that reported spontaneous pain to 500 μM S1P, we also observe that elevated S1P (2 μM or higher) triggers spontaneous pain in the cheek model (Figure 2C). While this other study did not measure mechanical or heat hypersensitivity after S1P

injection, our new data now show that the spontaneous pain behaviors induced by elevated S1P are due to heat and not mechanical hypersensitivity and that hypersensitivity is dependent on S1PR3 (Figure 2A-B, D). Overall, our data reveal two key, novel findings. First, S1P/S1PR3 sets baseline mechanical pain thresholds. Second, elevated S1P levels, such as those produced during inflammation or disease, promote heat hypersensitivity, but not mechanical hypersensitivity.

In addition to clarifying our results in the context of these previous findings throughout the revised manuscript, we have also added a significant section to the discussion that puts our results in context with past studies:

“Previous studies of S1P signaling in DRG neurons focused on S1P-evoked excitation of small diameter and/or capsaicin-sensitive neurons, and pain behaviors triggered by elevated S1P. [...] Our observation that S1PR3 KO animals display normal immune cell infiltration and develop mechanical hypersensitivity after CFA is consistent with previous studies showing distinct mechanisms of inflammatory thermal and mechanical hypersensitivity.”

...[D]emonstration using ISH of mRNA in many DRGS neurons (which included a nice use of SPIR3-/- mice as a control, just as in the current manuscript).

Unfortunately, we cannot make direct comparisons of our data to theirs, as they did not examine co-localization with other markers. And although they conclude that *S1pr3* is expressed by “virtually all” somatosensory neurons, they did not quantify *S1pr3* expression in wild type ganglia. Regardless, we now provide extensive new data, which, combined with our previous data, supports selective expression of S1PR3 in two neuronal subsets: mechanonociceptors and thermal nociceptors.

We have now performed a thorough ISH characterization to better characterize *S1pr3*-expressing cell types, including the use of new markers. These data show there are two main populations of *S1pr3*⁺ neurons: *Scn1a*⁺ mechanonociceptors (39.9% of *S1pr3*⁺), and *Trpv1*⁺ and/or *Trpa1*⁺ (67.1%) thermal nociceptors (Figure 3A-B). Staining of sectioned DRG, which is now included, shows that S1PR3 overlaps with Peripherin-expressing small-diameter neurons and IB4⁺ nociceptors as well as NF200+ medium-large myelinated neurons (Figure 3C,F).

Our whole mount and sectioned skin staining suggests that S1PR3 is not expressed in Merkel afferents or NefH⁺ hair follicle nerve endings (Figure 3D-E), which comprise subsets of LTMRs, but is rather expressed in NefH⁺ and NefH^{free} nerve endings, which comprise AMs and C nociceptors, respectively.

2) Also, the authors fail to mention a series of papers from the Nicol lab showing involvement of SIP and S1PRs in pain, including showing that SIP enhances excitability of DRG neurons by reducing a potassium current and also enhancing TTX-resistant sodium current (Zhang et al., 2006) and that this effect is diminished using siRNA targeted to SIPR1 and SIPR2 receptors (Li et al., 2015).

Our current work represents the first analysis of S1P signaling in AM nociceptors, and is distinct from work performed in these previous papers, for a number of reasons. All previous studies of S1P signaling in DRG neurons, including those cited above, focused on small diameter and/or capsaicin-sensitive neurons. While our new data affirms the effects in thermal nociceptors observed by others (Figure 4B-E), our manuscript highlights a novel effect of S1P in modulation of rheobase and M current in mechanonociceptors. Moreover, the effects of S1P on excitability of mechanonociceptors are completely lost in the S1PR3 knockout (Figure 5A). We have added the following passages to the Results and Discussion to clarify this:

“We next interrogated the molecular mechanism by which S1P signaling in AM nociceptors may regulate mechanical pain. [...] Additionally, S1P significantly reduced slow, voltage-dependent tail current amplitudes (Figure 5D; Figure 5E (top)) in an S1PR3-dependent manner (Figure 5D, center).”

“Previous studies of S1P signaling in DRG neurons focused on S1P-evoked excitation of small diameter and/or capsaicin-sensitive neurons, and pain behaviors triggered by high S1P. While our new data affirms the effects S1P in thermal nociceptors observed by others, our manuscript highlights a novel effect of S1P in modulation of rheobase and KCNQ2/3 currents in mechanonociceptors.”

3) A second major deficiency is in the analysis of the ionic mechanism underlying the increase in excitability reported here (and in the previous papers) by S1P applied to DRG neurons. The authors attribute this to closure of Kv7 channels (M-current). The evidence for this particular mechanism is very weak. The standard protocol for M-current is recording the slowly-deactivating outward tail currents when stepping from a steady holding potential of around -40 mV or -30 mv to around -60 mV. Here, the authors measure tail currents at -80 mV following steps to +100 mV. It is very hard to interpret these currents. It does not make sense to try to measure KV7 currents repolarizing to -80 mV, because the driving force on K is so small. In any case, I could not understand in Figure 5D how the authors were making the measurements or what the currents are. There is a sequence of fast-decaying current followed by a slowly-increasing outward current. This looks nothing like a recording of M-current (which would be a monotonically slowly-decaying outward current). The only evidence for it being mediated by Kv7 channels is occlusion by 100 μm linopirdine. This is of doubtful selectivity. The standard inhibitor of M-current (Kv2/Kv3) is XE-991, which acts with an IC50 of less than 1 uM.

This attribution of the increase in excitability by S1P to Kv7 inhibition is all the more unconvincing in light of the previous work done, which is not mentioned. Zhang et al. (2006, above) found that S1P both inhibited a high-threshold voltage-activated K current (clearly not Kv7 from its voltage dependence) and also enhanced TTX-resistant sodium channels, and Camprubi-Robles found that S1P induces a large inward current at holding potential of -80 mV (clearly not closing Kv7 channels, which would already be

(closed at this voltage), which they attributed to activation of chloride channels. The overall impression from all the work is that multiple conductances are affected. The evidence for closure of Kv7 channels being important is probably the least strong of any of the conductances implicated.

We have performed new experiments that address these concerns. While we originally reported results using linopirdine and +100 to -80 mV voltage steps, we also performed experiments with the suggested M current recording protocol (-40 to -60 mV steps). Using this protocol, the effects of S1P on M current were completely occluded by XE-991 (Figure 5E). These results dovetail with previous studies showing that KCNQ2/3 channels mediate M currents in cultured A-delta neurons, which include AM nociceptors and D-hairs (Schutze et al. 2016; Crozier et al. 2013), and support our model wherein S1P/S1PR3 constitutively modulate M currents in AM nociceptors.

In regard to the effects described by the aforementioned studies on thermal nociceptors (Figure 4B-E), we do not observe S1P-evoked excitation of or changes in sodium or steady-state potassium currents in mechanonociceptors (Figure 5-figure supplement 1C-E). These additional concerns are addressed above (see response to Reviewer 3 comment 1). See also our added results regarding S1P-evoked inhibition of KCNQ2/3 currents in mechanonociceptors:

“As tail currents in A δ neurons are primarily mediated by KCNQ2/3 potassium channels (30, 31), we postulated that S1P may alter tail currents through modulation of these channels. [...] In summary, our electrophysiological and behavioral observations support a model in which baseline S1P/S1PR3 signaling governs mechanical pain thresholds through modulation of KCNQ2/3 channel activity in AM neurons (Figure 8).”

[Editors' note: the author responses to the re-review follow.]

1) The new experiments with a conventional voltage protocol for M-current are convincing, and the new panels in Figure 5 are very nice. However, now it is hard for the reader to understand Figure 5C and 5D carried over from the previous version, or what the difference in protocol was for the "tail currents" plotted in Figure 5C and D versus those in Figure 5E. The voltage protocols for Figure 5C and Figure D are not shown, and it is not explained where tail current was measured. This should be clarified in the Figure legend (based on the previous version, presumably currents were activated by steps to +80 or +100, but I was unclear then and am still unclear at what time after the repolarization to -80 mV the tail currents were measured.). The figure might now work better leaving out Figure C and D (or moving them to the Supplementary figure) but at the least the protocols and measurements need to be explained clearly.

We thank the reviewers for the suggestion to clarify Figure 5. For Figure 5C, used an IV protocol whereby we recorded the current from -100 to +80mV in incremental 20mV steps, before and after S1P application. We have now clarified this in the figure legend as follows:

“C. The S1P-sensitive current is carried by potassium. The current-voltage relationship was determined by subtraction of the post-S1P current from the pre-S1P current and reverses at -60.125 mV; N = 6 cells. Data were fitted with a Boltzmann equation. Pre- and post-S1P currents were measured at the indicated voltage (-100mV to +80mV, 20mV increments) following a +100 mV step (100 ms). Current was quantified using the peak absolute value of the slowly-deactivating current 0-10 ms after stepping to indicated voltage. Unless indicated otherwise, all error bars represent mean \pm SEM. D. (Graphic, top) Averaged current traces of a single mCherry+ neuron in whole cell voltage clamp recording comparing tail currents (ΔI_{tail}) pre- and post-S1P using indicated voltage step protocol. (graphic, bottom) Averaged current traces of a single mCherry+ neuron in whole cell voltage clamp recording with XE991 treatment. Holding phase (-40 mV, 150 ms) was truncated in traces. (Left graph)% Δ in outward tail current (average +/- SD after indicated treatments (1 μ M S1P, 3 μ M XE 991, or both) for S1pr3^{mCherry/+} medium-diameter neurons; (p = 0.58; one-way ANOVA; n = 6, 8, 14 cells) using protocol depicted at right. (Right graph)% Δ in inward tail current after indicated treatments (LINO = 100 μ M linopirdine) for S1pr3^{mCherry/+} medium-diameter neurons; (p = 0.47; two-tailed paired t-test; N = 12 cells).”

As suggested, we have also moved Figure 5D to Figure 5—figure supplement 1F. The supplemental figure legend now reads:

“F.% Δ in inward tail current (ΔI_{tail}) after S1P or 1% DMSO vehicle application for S1pr3^{mCherry/+} and KO medium-diameter neurons using a pre-pulse stimulation of +80 mV followed by a step to -80 mV, where (ΔI_{tail}) was calculated by subtracting the steady-state current from the absolute peak of the slowly-deactivating current at -80 mV (p = 0.014; one-way ANOVA; N = 10, 13, 10 cells). Tukey Kramer post hoc p-values indicated on graph.”

2) It was hard to understand Figure 6E until going deep into Materials and methods section. It is not at all obvious from the traces in the hets why the top is classified as "non-adapting" and the bottom as "adapting". Both keep firing throughout the stimulus and both show much slower firing in the second half of the stimulus. In fact, the firing during the later stimulus is faster in the "non-adapting" fiber than in the "adapting" fiber, which seems confusing. One has to go to the Methods to realize that the distinction is when frequency falls by 2-fold from the initial frequency to the end, so the initial frequency must be much faster in the bottom trace than the top. It would be much clearer if the plots included a trace of frequency averaged over 200 ms intervals or something similar. If that doesn't fit well into the figure, at least the main text should explain the criterion that is being used, and maybe say something like "the top trace shows an example of a non-adapting fiber, in which the initial frequency of xx Hz fell less than 2-fold over the 5-sec stimulus, to xx Hz. The bottom trace shows an example of a non-adapting fiber, in which the initial frequency of xx Hz fell to xx HZ by the end of the stimulus."

We thank the reviewers for the suggestion to clarify Figure 6E and the corresponding main text. We have updated the figure to include the binned instantaneous firing-rate frequency and the caption now reads:

“E. Representative traces and binned instantaneous firing frequencies (IFF; 200-ms bins) of Non-Adapting and Adapting AMs in response to force controlled stimulation (256 mN, top) for S1PR3 HET and KO mice; blue regions, dynamic phase of stimulation (200-ms).”

We also modified the associated main text as follows (changes underlined):

“A recent study reported that A-nociceptors are composed of two genetically distinct neuronal populations that differ in conduction velocity and in adaptation properties⁵ (“Adapting AM” versus “Non-adapting AM”). We next asked whether loss of S1PR3 signaling altered these AM subtypes. Adapting AM fibers responded more vigorously to dynamic (ramp) stimuli than static (hold) stimuli, and displayed a mean dynamic firing frequency at least twofold greater than their static firing frequency⁵ (Figure 6E, upper traces). By contrast, Non-adapting AM fibers often showed bursting during static stimulation, which resulted in similar firing rates during dynamic and static stimulation (Figure 6E, lower traces). S1PR3 KO animals displayed a significantly lower proportion of Adapting AM nociceptors compared with littermate controls (Figure 6F). Additionally, we observed an increase in S1PR3 KO AM fibers that were unresponsive to controlled force stimulation (Figure 6F). These “non-responders” only fired action potentials to high-pressure stimuli with a blunt glass probe or to suprathreshold stimulation with von Frey filaments (see Materials and methods section). The Non-Adapting AMs, and the few remaining mechanosensitive Adapting AMs in the S1PR3 KO displayed similar firing frequencies over both the dynamic and static phases of force application to control fibers (Figure 6—figure supplement 1C). This suggests that decreased mechanosensitivity of the Adapting AM population accounts for the significant reduction in force-firing relations observed at the population level in S1PR3 KO AMs (Figure 6A). We conclude that S1PR3 is an essential regulator of both mechanical threshold and sensitivity in a distinct population of AM nociceptors.”

Additionally, we have included a supplemental figure (Figure 6—figure supplement 1C) to clarify the phenotype observed in S1PR3 KO Adapting AMs that is described by the new main text. The figure caption reads:

“C. Mean firing rates during dynamic (ramp) and static (hold) stimulation for S1PR3 HET and S1PR3 KO recordings (left, Adapting AMs; right, Non-Adapting AMs; see Figure 6E-F for experimental details). No significant differences were found between genotypes ($p = 0.227, 0.490$ (two-way ANOVA); bars, means). As shown in Figure 6F, the proportion of Adapting AMs was significantly lower in S1PR3 KO recordings compared with littermate controls.”

3) *Figure 5—figure supplement 1E. "(Left)% Δ in peak instantaneous sodium current afterSIP" – one assumes that it isn't "instantaneous" current but just "peak sodium current" during the depolarization that is plotted.*

The figure legend now reads “% Δ in peak sodium current”, as requested.

4) Subsection “*Endogenous S1P mediates acute mechanical pain*” “that increased of S1P does not evoke...” – delete “of”.

This portion of the main text now reads “that increased S1P does not evoke...”.

Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Supplementary Materials

Figure 1—source data 1. S1PR3 mediates acute mechanical pain.

Related to [Figure 1](#).

[eLife-33285-fig1-data1.xlsx](#) (42.7KB, xlsx)

DOI: 10.7554/eLife.33285.004

Figure 2—source data 1. Endogenous S1P mediates acute mechanical pain.

Related to [Figure 2](#).

[eLife-33285-fig2-data1.xlsx](#) (49KB, xlsx)

DOI: 10.7554/eLife.33285.006

Figure 5—source data 1. S1PR3 modulates KCNQ2/3 channels to regulate AM excitability.

Related to [Figure 5](#).

[eLife-33285-fig5-data1.xlsx](#) (43.5KB, xlsx)

DOI: 10.7554/eLife.33285.012

Figure 6—source data 1. S1PR3 is required for nociceptive responses of high-threshold AM nociceptors.

Related to [Figure 6](#). Table provides properties of all S1PR3 HET and KO AM fibers that were recorded.

[eLife-33285-fig6-data1.xlsx](#) (27.9KB, xlsx)

DOI: 10.7554/eLife.33285.015

Figure 7—source data 1. S1PR3 is dispensable for development of chronic mechanical hypersensitivity.

Related to [Figure 7](#).

[eLife-33285-fig7-data1.xlsx](#) (44.3KB, xlsx)

DOI: 10.7554/eLife.33285.018

Supplementary file 1. Co-ISH quantification for sectioned DRG from adult wild-type mice.

Related to [Figure 3](#).

[elife-33285-suppl1.xlsx](#) (37.6KB, xlsx)

DOI: 10.7554/eLife.33285.020

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